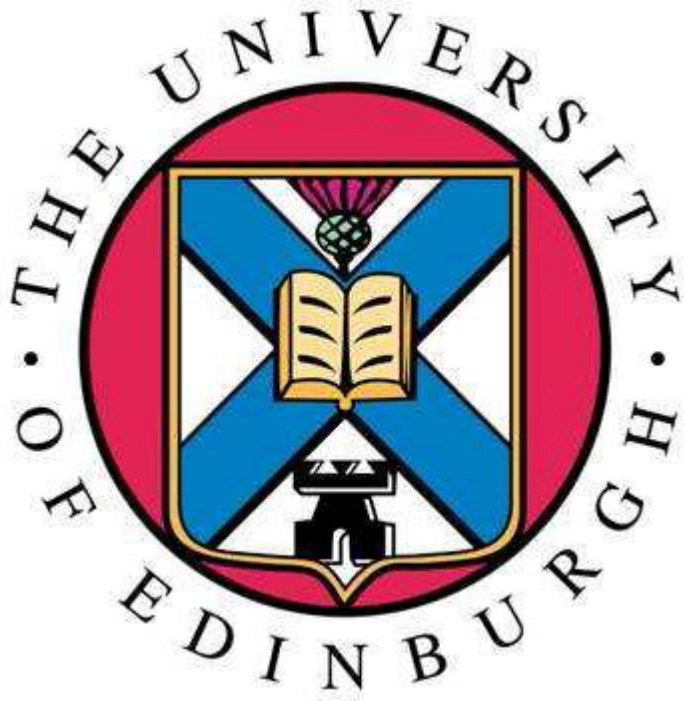
THE UNIVERSITY *of* EDINBURGH

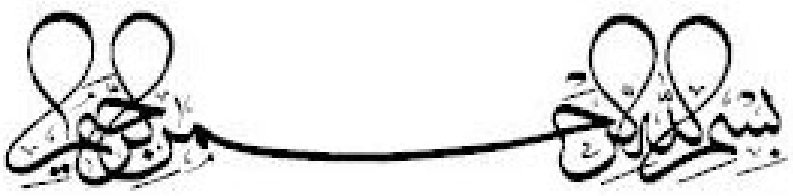


Characterization of CTX-M β-lactamases in Enterobacteriaceae from a major teaching hospital

Maher Sulaiman M Alqurashi

Thesis presented to the University of Edinburgh for the degree of PhD

2013



In the name of Allah the most Gracious the most Merciful

Abstract

Escherichia coli and *Klebsiella pneumoniae* cause a wide range of infections. Multidrug-resistance strains carrying extended-spectrum β-lactamases (ESBLs) has become a growing problem worldwide. The CTX-M type ESBLs has emerged distinctly, especially in *Escherichia coli* and *Klebsiella pneumoniae*. CTX-M type has been associated with many outbreaks of infections both in the hospitals and community. CTX-M-15 is now identified as the most predominantly distributed CTX-M enzyme. Clonal outbreaks of CTX-M-15 producing Enterobacteriaceae have been described in many countries including the United Kingdom, and *Escherichia coli* is the most commonly involved species.

A total of 100 isolates were received in 2010 from London St George's hospital, England, 50 *Escherichia coli*, 17 *Klebsiella* spp, 9 *Enterobacter* spp, 13 *Proteus* spp, 6 Lactose fermenting coliforms, 2 *Pantoea* spp, one *Serratia marcescens*, one *Morganella morganii*, and one *Hafnia alvei*. The antimicrobial susceptibility results showed that 5 *Escherichia coli* and one *Klebsiella pneumoniae* isolates were found to be resistance to cefotaxime, ceftazidime, ceftriaxone, cefotaxime, , ciprofloxacin, , and gentamicin, making them multi-drug resistant bacteria. None of the isolates showed resistance to imipenem, ertapenem, or morepenem, thus making carbapenems the drug of choice for the treatment of these infections due to multi-resistant isolates. The overall frequency of CTX-M-15 type ESBL-producers detected in this study was 6 (6%) most of them 5/6 (83%) were from *Escherichia coli* and one was (17%) *Klebsiella pneumoniae* isolates. The 6 CTX-M-positive isolates were typed by PFGE, only two strains of *Escherichia coli* showed more than 85%

similarity, owing to clonal homology for both strains. The rest strains showed less than 85% similarity.

S1 nuclease plasmid profiles were obtained for ESBL-producers isolates. A total of one to three plasmids per isolate, ranging from approximately 78.0 to 152.0 kb, were observed. The plasmids from most isolates were assigned to be IncFA and IncFB replicons. Analysis of phylogenetic groups showed group A and group B2. The method of phylogenetic classification of exteraintestinal pathogenic *Escherichia coli* depends on examine and combination of two preserved genes (*chuaA* and *yjaA*) and the DNA fragment TSP.

Primer walking and PCR experiments were used for the genetic environment studies which showed 5 different genetic constructions for the described $bla_{\text{CTX-M-15}}$ genes. Conjugation studies were used to detect the transferability of the plasmids harbouring the reported $bla_{\text{CTX-M-15}}$ genes. Three isolates were found transferable by conjugation.

In conclusion, this study reports the presence of hospital highly resistant $bla_{\text{CTX-M-15}}$ in St George's hospital. The spread of $bla_{\text{CTX-M-15}}$ is probably due to horizontal gene transfer harbouring IS*Ecp1* and the conjugative properties of plasmids carrying $bla_{\text{CTX-M-15}}$.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

No portion of the work referred to in this thesis has been submitted in support of an application for another degree.

Maher Sulaiman M Alqurashi

Acknowledgments

First and foremost, thank you to ALLAH and it is time to express my gratitude to all those who gave me the possibility to complete this thesis. I would like to say a big thank you to them all.

My supervisors Professor Sebastian Amyes, Dr. Ahmed Hamouda, Dr. Catherine Doherty, for guiding me and teaching me everything they could and providing me the opportunity to work with under guidance.

Special thanks for my supervisor Sebastian Amyes for correcting my mistakes, making me learn how to be a true scientific researcher, and for setting me a wonderful example of true, kind and great mentor.

My parents, since I was born they always been with me step by step, they dedicated their life for me, always prayed for me. I would say this thesis would not exist without you, and I love you in every second of my life.

My wife, Asmaa, thank you my love, words cannot express my feeling, but you know without you I am nothing.

My children, Mhannad, Jumanah, Omar, Amar, Banan, and Bayan, you are my treasure in this life and thank you for enriched my life.

My colleagues who helped me and shared good and bad times together.
Eventually, I thank ALLAH again for everything.

Paper presented at the 22nd ECCMID, London 2012

M.S. Alqurashi, J. Dave, M. Pond and S.G.B. Amyes

The role of plasmids in the control of antibiotic resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolated from St George's Hospital London

Objectives: The aim of this study was to investigate the infiltration of extended-spectrum β-lactamases (ESBLs) and their association with individual clones of Enterobacteriaceae isolated from blood cultures within a London teaching hospital.

Methods: One hundred Enterobacteriaceae, isolated sequentially in August - October 2009 from blood culture specimens, were obtained from St George's Hospital London. Antimicrobial susceptibility testing was determined by disc diffusion and agar dilution was used to measure the MICs of Cefotaxime, Ceftazidime, and Ceftriaxone; all tests were interpreted according to the BSAC guidelines. Multiplex PCR was used to identify CTX-M and AmpC enzymes. Pulsed-field gel electrophoresis (PFGE) with *Xba1* restriction was used to genotype the strains. Plasmid profiles were determined using S1 nuclease and alkaline lysis extraction and examined by PFGE. Furthermore, PCR based replicon typing was used to identify individual plasmid types.

Results: Six isolates showed resistance to cefotaxime, ceftazidime and ceftriaxone, five were *Escherichia coli* and one was *Klebsiella pneumoniae*. All of them possessed the CTX-M-15 β-lactamase gene but none of the strains harboured any of the known transferable AmpC β-lactamases. Genotyping by PFGE revealed that two of the CTX-M-15 β-lactamase-containing isolates of *Escherichia coli* were closely related but the remaining isolates were less than 80% identical. In the six isolates, the plasmid profiles were not identical, ranging from 97.0kb to 145.5kb though four isolates of *Escherichia coli* did have the same plasmid of 145.5kb.

Conclusion: Our data indicate the presence of plasmids carrying the CTX-M-15 β-lactamase within this Enterobacteriaceae population and suggest that the dissemination of CTX-M-15 β-lactamase gene is linked to both the transfer of these genes between plasmids and the transfer of those plasmids between the individual strains.

Published in Clinical Microbiology and Infection (2012) 18: S333.

Abbreviations

Å	Angstroms
A. baumannii	*Acinetobacteri baumannii*
B	β
bla	β-lactamase
BLAST	Basic Local Alignment Search Tool
BSAC	British Society of Antimicrobial Agents and Chemotherapy
CLSI	Clinical Laboratory Standards Institute
CTX-M	Cefotaxime
DNA	Deoxyribonucleic acid
E. aerogenes	*Enterobacter aerogenes*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-spectrum-β-lactamase
EUCAST	European Committee on antimicrobial susceptibility testing
GW-PCR	Genome walking-polymerase chain reaction
Inc	Incompatibility
K.	*Kluyvera*
Kb	Kilo-base pairs
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre
MBLs	Metallo-β-lactamases
MDR	Multi-drug resistance/resistant
Mg	Milligram
MIC	Minimum inhibitory concentration
mL	Milli-Litre
mM	Milli-Molar
M	Molar
MGE	Mobile genetic elements
µ	micro
µg	microgram
NCBI	National Centre of Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Measure of acidity or basicity of a solution
PK	Proteinase K
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
Spp.	species
TAE	Tris/acetic acid/ethylenediaminetetraacetic acid
TBE	Tris/Borate/ethylenediaminetetraacetic acid
TE	Tris/EDTA
U	Units
UTI	Urinary tract infection

V	Volts
w/v	Weight by volume

Contents

List of tables

List of figures

Section A

CHAPTER-1: General Introduction

1.1. Antimicrobial agents

1.1.1 Definition

The term antibiotic has traditionally indicated to natural metabolic products of fungi, actinomycetes, and bacteria that inhibit or kill the growth of microorganisms. Antibiotic production has been particularly linked with soil microorganisms and in the natural environment is believed to provide a selective advantage for organisms in their competition for nutrients and space. While the majority of antimicrobial agents in clinical use today are made from natural products of fermentation, most are then modified chemically (semi-synthetic) to improve their antibacterial or pharmacologic properties. Nonetheless, some agents are totally synthetic such as sulphonamides and quinolones. Therefore the name antibacterial or antimicrobial agent is often used in preference to antibiotic (Greenwood, 2000).

1.1.2. Historical prospective

Antimicrobial agents have been used for the treatment of infectious diseases since the 17th century such as quinine for malaria (Hien & White, 1993) and emetine for amebiasis (Knight, 1980). during the first decade of 20th century, Paul Ehrlich was the first to propose the relation between microbial pathogens and drugs, as his experiments led to the arsphenamines for the treatment of syphilis (Montes & Cueva, 1967). The present era of antimicrobial chemotherapy began in 1935, with the discovery of the sulphonamides by Gerhard Domagk (Otten, 1986). In 1929, penicillin was discovered by Alexander Fleming and he proposed that penicillin could be an effective chemotherapeutic agent (Fleming, 1929). However, Fleming was unable to exploit this clinically and it was left to Florey and Chain to purify penicillin and prepare it for clinical use in 1940 (Chain & Florey, 1940). Penicillin was only active against Gram-positive bacteria and it was not until Selman Waksman discovered streptomycin from soil bacterium *Streptomyces griseus* in the late 1940s, so Gram-negative bacteria became treatable with antibiotics (Schatz *et al*, 1944**)**. From this discovery, many new classes of antibiotic had been described by the 1960s (Kingston, 2004). The semisynthetic penicillins introduced by Beecham in 1960s included methicillin, ampicillin and cloxacillin. Meanwhile, the cephalosporin C was established by Abraham and Newton (Rolinson, 1998).

1.1.3. Modes of action

Antimicrobial drugs have several mechanisms (Brooks 2007; Amyes 2010) include:

i) Interference with cell wall synthesis such as β-lactam antibiotics now include: penicillinase-resistant, amino-, carboxy-, indanyl-, and ureidopenicillins; first- to fifth-generation cephalosporins; monobactams; and carbapenems.

ii) Interference of protein synthesis: Aminoglycosides, Macrolides, Tetracyclines, Lincomycins, Chloramphenicol, Linezolid, Streptogramins.

iii) Interference with nucleic acid (DNA) synthesis by interfering with DNA gyrase and topoisomerase IV: Quinolones, Metronidazole.

iv) Inhibition of Ribonucleic acid (RNA) synthesis by acting on DNAdirected RNA polymerase: Rifamycins.

v) Inhibition of a metabolic pathway by acting on the synthesis of tetrahydropholic acid: Trimethoprim, Sulfamethoxazole.

vi) Disruption of bacterial membrane structure: Polymyxins.

1.1.4. Mechanisms of resistance to antimicrobials

1.1.4.1. Intrinsic resistance

The inherent resistance of bacteria to an antimicrobial may be expressed as a result of general adaptive processes that are not associated to a specific class of antimicrobials. For example, the natural low membrane permeability of *Pseudomonas aeruginosa*is is most likely due to of its innate resistance to many

antimicrobials (Yoneyama & Katsumata, 2006). Other examples of intrinsic resistance are the outer membrane of Gram negative bacteria, the presence of genes giving resistance to self-produced antibiotics, general absence of the target hit by the antimicrobial or absence of bacterial uptake transport system for the antimicrobial ((Normark and Normark, 2002)).

1.1.4.2. Acquired resistance

Acquired resistance causes most concern. Initially, a bacterial population may be susceptible to an agent then it acquires resistance under the selective pressure of that agent. Bacteria use several mechanisms to confer resistance, which then spread to a variety of bacterial species and genera (Wright, 2005). This active resistance includes three mechanisms: First, the bacteria may acquire genes encoding enzymes that destroy the antibacterial agent before it can act; an example of this is the β-lactamases (Abraham & Chain, 1940). Second, bacteria may possess efflux pumps that remove the antibiotic agent from the cell before it can bind to the target site (Levy & McMurray, 1974). The third, bacteria may possess genes for a metabolic by-pass pathway which creates na altered target; in the case of trimethoprim this would be an altered dihydrofolate reductase (Amyes & Smith, 1974) or, for the cell wall, an altered terminal residue on the peptidoglycan pentapeptide that is not capable of binding glycopeptides (Arthur & Courvalin, 1993). Finally bacteria may also limit the access of antibacterial agents by mutations in genes that regulate porins (Yoneyama & Katsumata, 2006; Wright, 2005).

Bacteria may develop resistance by the acquisition of new genetic elements from other resistant bacteria; this termed horizontal evolution may take place between strain of the same species or different species and genera. Mechanisms of genetic exchange include conjugation, transduction and transformation (Thomas & Nielsen, 2005).

Mutations and selection, together with the genetic exchange mechanisms, may enable bacterial species to adapt rapidly to the introduction of antibacterial drugs into their environment. However, a single mutation may be sufficient for the bacteria to survive until they acquire additional mutations or additional genetic materials resulting in full resistance to the antimicrobial agent (Bushman, 2002; White *et al*, 2005).

1.1.5. β-lactam antibiotics

1.1.5.1. Structure

The β-lactam antibiotics include penicillins, cephalosporins, carbapenems, and monobactams. Their name comes from the presence of a β-lactam ring in their structure; this ring is vital for antimicrobial activity (Sherris, 2004) (Figure 1.1). In 1940, Florey and Heatley travelled to the United States seeking help in mass production of penicillin by fermentation (Richards, 1943). They were able to isolate Penicillin from *Penicillium chrysogenum*. This work attracted pharmaceutical firms to invest in production of penicillin in enormous amount (Chain, 1979). Thereafter, different penicillin compounds were introduced by E.R. Squibb & Sons (Chain,

1979) and Florey's group (Queener *et al*, 1986) producing benzyl-penicillin (Penicillin G) and 2-pentenylpenicillin respectively (Penicillin F) (Abraham *et al*, 1949). It was later observed that both of penicillin forms contained β-lactam rings (Figure 1.1).

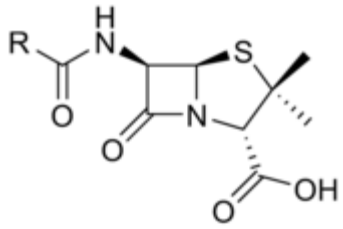


Figure 1.1: The structure of Penicillin nucleus. β-lactam ring is the square at the centre

Scientists realized that early work applying different fermentation conditions were not that successful (Behrens *et al*, 1948). The β-lactam nucleus, which is 6-aminopenicillanic acid (6-APA), proved to be the essential part of penicillin synthesis and modification. Therefore, Shaheen & Henery (1959) used acylation of chemically synthesized 6-APA to produce Penicillin V. This work the opportunity for new β-lactam agents to be produced by adding side chains to 6-APA. Subsequently, semi-synthetic β-lactam agents have been progressed continuously and systemically; for example, methicillin was the first semi-synthetic penicillin released in 1960 to resist hydrolysis by penicillinases (Fairbrother & Taylor, 1960) (Figure 1.2). The use of natural sources continued to be searched through the production of

semi-synthetic agents. Cephalosporin C was isolated from *Cephalosporium acremonium* by Abraham and Newton (Abraham & Newton, 1961). This agent produced a novel family of β-lactam antibiotic where it possesses a nucleus of 7-aminocephalosporinic acid instead of 6-APA (Figure 1.3). Cephalosporin generations with effective broad spectrum activity have been released using 7-ACA as the precursor and isolated from bacteria. These include monobactam from *pseudomonas acidophia* (Imada *et al*, 1981), *Chromobacterium violaceum* (Skyes *et al*, 1981), and *Agrobacterium radiobacter* (Well *et al*, 1982), and carbapenem from *Streptomyces spp (*Nagarajan *et al*, 1971). Carbapenems, such as imipenem and meropenem have become the antibiotic of choice for treatment of most serious bacterial infections, owing of its broadest spectrum of aerobic and anaerobic activity (Livermore & Woodford, 2006). The structures of carbapenems and penicillins chemically are similar except the sulphur atom in position 1 of the penicillin structure has been substituted with a carbon atom (Figure 1.4) (Kumagai *et al*, 2002).

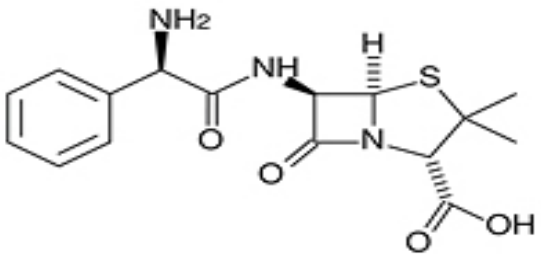


Figure 1.2: Methicillin structure shows the side chain attached to the β-lactam ring producing semi-synthetic β-lactam compound.

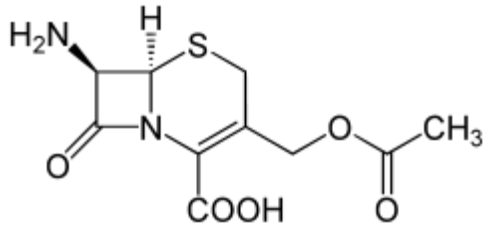


Figure 1.3: Cephalosporin C antibiotic shows 7- aminocephalosporinic acid

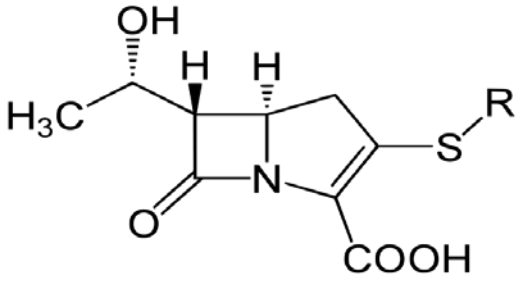


Figure 1.4: General structure of carbapenems

1.1.5.2. Mechanism of action of β-lactam antibiotics

Bacteria depend on a heavily cross-linked peptidoglycan layer in the cell wall for the protection of cell shape and rigidity. Bacterial cell walls comprise a basic repeating unit of alternating disaccharides; N-acetyl glucosamine and N-acetyl muramic acid. The former sugar is modified by a unique pentapeptide. The composition of this pentapeptide varies between the Gram-positive and Gram-negative species, but always ends in two D-alanine residues. The individual peptidoglycan units are created inside the bacterial cell, but their final cross-linking is catalysed outside the

bacterial cytoplasmic membrane by a group of membrane anchored bacterial enzymes called the cell wall transpeptidase (Fisher *et al*, 2005; Wilk *et al*, 2005). In cross linking reaction, a peptide bond is built between the penultimate D-alanine on one chain and the free amino end of an L-lysine for Gram positive or a diamino pimelic acid for Gram negative residue on the other chain. The linkage is made with the penultimate D-alanine, resulting in the terminal D-alanine to be split in the process (Tipper & Strominger 1965; Ghuysen, 1988; Livermore & Williams, 1996; Wilk *et al*, 2005).

β-lactam antibiotics inhibit the bacterial transpeptidase enzymes that use an active site serine and carry out their catalytic cycle. The later enzymes are referred as penicillin binding proteins (PBPs) (Spratt, 1994). The β-lactam drugs are able to do this, because they have stereochemical similarity with the D-alanine-D-alanine substrate. In the presence of penicillin, the transpeptidases create a lethal covalent penicilloyl-enzymes complex that aids to block the normal transpeptidation reaction. Consequently, weak cross linked peptidoglycan will occur, which induces the growing bacteria highly capable to cell lysis and death (Wilke *et al*, 2005).

1.1.5.3. Mechanisms of resistance to β-lactam antibiotics

β-lactam antibiotics perform their action by binding to the PBPs, thus inhibiting the synthesis of peptidoglycan. Inhibition of PBPs weakens the bacterial cell wall, resulting in cell growth inhibition and eventually leading to cell death. There are

three main mechanisms of β-lactam resistance; decreased access to the PBPs, decreased PBP binding affinity, and destruction of antibiotic by the expression of β-lactamase which can bind and hydrolyse β-lactams (Ambler, 1980).

1.1.5.3.1. Penicillin binding protein (PBP) modifications

Because β-lactam antibiotics can inhibit peptidoglycan synthesis in all bacteria that possess a cell wall, a certain target must be present for these antibiotics. The existence of a specific PBP can be detected by mixing cell membranes with a radioactively labelled β-lactam compound, followed by electrophoretic separation to identify the protein species to which the β-lactam is covalently bound. All bacteria have several proteins that bind covalently with penicillin but have different affinities and specificities for various β-lactams. Three to seven PBPs may be present in any species having different enzymatic activities and may contain glycosyltransferases, peptidyltransferases, transpeptidases, endopeptidases and D-alanine carboxypeptidases (Frere *et al*, 1992).

In *Escherichia coli*, there are seven major PBPs, each one of which can be inactivated by mutation or by treatment with a β-lactam having a high affinity for that PBP (Spratt, 1975; Spratt, 1977). PBP5 and PBP6 are present in the largest amounts and their carboxypeptidase activity hydrolyses the terminal D-alanine from the newly incorporated peptidoglycan pentapeptide; however neither enzyme is essential for cell growth (Spratt, 1980; Broome-Smith, 1985; Baquero *et al*, 1996). PBP3 is involved in septum formation and the β-lactams (ampicillin and cephalexin), with the highest affinity for PBP3, cause in the formation of long filaments or bulged cells that lack a division septum (Spratt, 1975; Botta *et al*, 1981). PBP2 appear to help determine cell shape because mutants lacking this activity or wild type cells inhibited by a β-lactam antibiotic specific for PBP2, form viable, ovoid shaped cells

(Spratt, 1975; Spratt & Pardee, 1975; Ogura *et al*, 1989). Treatment of *Escherichia coli* with a concentration of cephaloridine that will bind only PBP1B causes cell lysis, owing to the fact that this PBP is essential for synthesis of an integral cell wall and is the lethal target for this antibiotic. PBP1A and PBP1B also are blocked by cefsuloldin and are responsible for elongation or overall cell wall growth; these PBPs have both transpeptidase and transglycosylase activities. Therefore, various β-lactam antibiotics have the same mode of action by their covalent linkage to the enzymes responsible for modifying the newly incorporated peptidoglycan subunits. β-lactams can have different consequences for different bacteria owing of their preference for different PBPs, which carry out different enzymatic reactions or act at different sites in the growing cell wall (Suzuki *et al*, 1978; Yousif *et al*, 1985) .

Overall, the PBPs from most bacteria have similar affinities for β-lactams. The variation in susceptibilities of different bacteria to β-lactams usually is not a consequence of the PBPs themselves, but of differences in the effective concentrations of the antibiotics at their site of action. Primary factors that determine the β-lactam level at the growing wall are the rate of diffusion of the antibiotic through the cell envelope and the susceptibility of the antibiotic to β-lactamases produced by the bacterium or its neighbours (Spratt, 1975; Spratt, 1977; Tomasz, 1980; Handwerger & Tomasz, 1985).

1.1.5.3.2. Impermeability causing resistance

The ability of the bacterial outer membrane to exclude hydrophobic molecules is an unusual feature among biologic membranes and serves to protect the bacterial cell (in case of enteric bacteria) from bile salts. Because of its lipid nature, the bacterial outer membrane is able to exclude hydrophilic molecules. However, the outer membrane has special channels, containing of protein molecules, called porins that allow the passive diffusion of low molecular weight hydrophilic compounds such as sugars, amino acids, and ions. Large antibiotic molecules penetrate the bacterial outer membrane relatively slowly, which accounts for the relatively high antibiotic resistance of Gram negative bacteria (Silver, 2003; Yoneyama & Katsumata, 2006). The permeability of the bacterial outer membrane varies widely from one Gram-negative bacterium to another; for example, *Pseudomonas aeruginosa* is extremely resistant to antibiotics as the outer membrane is 100 times less permeable than that of *Escherichia coli* (Rachakonda & Cartee, 2004).

Some Gram negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have an innate low susceptibility to β-lactam drugs, a feature that is linked to reduced outer membrane permeability (Nikaido, 2003). The reduced permeability in *Pseudomonas aeruginosa* is due to a low number of porins, which also tend to be small and have different chemical features when compared to the porins of the Enterobacteriaceae (Nestorovich *et al*, 2006). In other Gram negative bacteria like *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli* and *Klebsiella* spp., β-lactam susceptibility is related to the existence of non-specific porins

belonging to the OmpC and OmpF groups (Davin-Régli & Pagès, 2006). Strains isolated from patients under antibiotic therapy, can express a characteristic reduction in cephalosporin and carbapenem susceptibility. A changed porin phenotype is also frequently associated with the exhibition of degradative enzymes such as β-lactamases, which provide a high level of β-lactam resistance (Nikaido, 1989; Pagès, 2004).

1.1.5.3.3. Efflux pump

Efflux pumps are transport proteins and have a role in the extrusion of toxic substrates such as antibiotics, from inside cells to the external environment. They can be recognised in both Gram-positive and Gram-negative bacteria and in eukaryotic as well (Bambeke *et al*, 2000). These pumps can be specific for one compound or can transport a range of structurally different compounds including antibiotics from a range of different classes. Such pumps may be associated with multiple drug resistance (MDR). Efflux pump as a mechanism of resistance was first described in the early 1980s for tetracycline (Ball *et al*, 1980). Thereafter, efflux pump-mediated resistance to many antimicrobial agents, has been identified in a variety of bacterial species, and a number of efflux pump determinants have been sequenced and cloned (Paulsen *et al*, 1996). There are five major families of efflux transporter in the prokaryotic kingdom: major facilitator (MF), multidrug and toxic efflux (MATE), resistance nodulation division (RND), small multidrug resistance (SMR), and ATP binding cassette (ABC) (Lomovskaya *et al*, 2001). Transporters that efflux multiple compounds, including antibiotics have not emerged in response to the antibiotic era.

It has been identified that 5% to 10% of all bacterial genes participate in transport and a large number of these encode efflux pumps (Saier & Paulsen, 2001), indicating that they must have preceded the antibiotic era.

There is a relation between resistance to bacteria and increase in gene expression of efflux pumps; for example; resistance to bile salts and antibiotics in *Escherichia coli* is caused by over expression of *acrAB* (Thanassi *et al*, 1997). Although genes encoding efflux pumps may be present on plasmids, the carriage of efflux pump genes on the chromosome confers the bacterium an intrinsic mechanism that permits survival in a hostile environment, such as the presence of antibiotics. Therefore mutant bacteria that over-express efflux pump genes, raising the threshold of resistance, may be selected without the acquisition of any new genetic elements. Probably these pumps emerged so that toxic substances could be excreted out of the bacterium. It is now widely acknowledged that the intrinsic resistance of Gram negative bacteria to specific antibiotics, compared with Gram positive bacteria, is due to the activity of an efflux system (Li, Livermore & Nikaido, 1994). Efflux systems that confer antibiotic resistance to some bacteria, have been identified from a number of clinically significant bacteria, including *Escherichia coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD9) (Poole, 2000), *Campylobacter jejuni* (CmeABC) (Lin, *et al*, 2000), *Pseudomonas aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) (Poole, 2000), *Staphylococcus aureus* (NorA) (Kaatz and Seo, 1995), *Streptococcus pneumoniae* (PmrA) (Gill *et al*, 1999), and *Salmonella typhimurium* (AcrB) (Nikaido, 2000). All of these efflux systems and the RND

pumps (CmeB, AcrB and the Mex pumps) also transport multiple antibiotics out of the bacterial cell membrane (Webber and Piddock, 2003).

1.1.5.3.4. Enzyme production

The most important mechanism of β-lactam resistance, especially amongst Gram negative bacteria, is the production of β-lactamases. These enzymes can hydrolyse β-lactam ring, resulting in the antimicrobial ineffective (Helfand & Bonomo, 2003). β-lactamase enzymes are structurally similar to PBPs (Massova and Mobashery, 1998) and may have emerged from β-lactam binding enzymes of cell wall biosynthesis. They were first described in *Escherichia coli* isolates before the release of the first β-lactam drug, penicillin (Abraham & Chain, 1940). Since then, these enzymes have been identified in Gram negative and Gram positive bacteria where they are found either chromosomally or plasmid encoded, and usually associated with mobile genetic structures such as transposons and integrons (Rowe-Magnus and Mazel, 2002).

By 2009, the number of individual protein sequences for β-lactamases was more than 890. β-lactamase enzymes production is most commonly suspected in Gram negative bacteria that exhibit resistance to a β-lactam antibiotic (Bush and Jacoby, 2010).

Classification based on molecular structure was first suggested by Ambler (1980) with two classes. Class A included PC1 β-lactamases encoded by the chromsome of *Staphylococcus aureus*, with a serine residue at the active site, and class B metallo-

β-lactamases including the type II enzyme encoded by *Bacillus cereus*, with Zn2+ at the active site. Jaurin and Grundstorm, (1981) identified class C cephalosporinases in *Escherichia coli* K12, with a serine residue at the active site. Class D β-lactamases were separated from other serine β-lactamases after sequencing of PSE-2 and OXA-1 hydrolysing carbencillin and oxacillin (Houvinen *et al*, 1988). Many kinetic, mutagenesis and structural reports have been done on these enzymes, contributing significant information about their substrate specificities and catalytic mechanisms (Helfand & Bonomo, 2003). Some of these enzymes can target the expanded spectrum β-lactams such as the cephalosporins, including the extended spectrum β-lactamases (ESBL) (classes A and D) (reviewed by Bradford, 2001), the AmpC (class C cephalosporinases) enzymes (Philippon, Arlet & Jacoby, 2002), and the carbapenemases that can hydrolyse most β-lactams agents, including the carbapenems (classes A, B and D) (Poirel & Nordmann, 2002).

The Ambler molecular scheme was modified to one based on a combination of both functional and molecular schemes. According to both systems classification, there are groups 1, 2, and 3, and subgroups 2a, 2c, 3a, etc (Bush & Jacoby, 2010). Group1 cephalosporinases which are not inhibited by clavulanate, group2 broad spectrum enzymes which comprise the largest category and are generally inhibited by clavulanate with the exception of the 2d and 2f sub-groups, and the group3 metallo-β-lactamases. However, most ESBLs are allocated to group 2be which can hydrolyse penicillins, monobactams, and cephalosporins and inhibited by clavulanate according to the Ambler classification. The CTX-M enzymes fulfill the criteria for group 2be enzymes (Dhillon and Clark, 2012) Table 1.1.

Table 1.1: β-lactamases classification schemes modified from (Bush and Jacoby, 2010).

Bush-Jacoby group	Ambler class	Main substrate	Inhibited by		Representative enzymes
			CA/TZB	EDTA	
1	C	Cephalosporins	-	-	AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	-	-	GC-1, CMY-37
2a	A	Penicillins	+	-	PC1
2b	A	Penicillins, early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	-	-	TEM-3, SHV-2, CTXMs, PER, VEB
2br	A	penicillins	-	-	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	-	-	TEM-50
2c	A	Carbencillin	+	-	PSE-1, CARB-3
2ce	A	Carbencillin, cefepime	+	-	RTG-4
2d	D	Cloxacillin	V	-	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	V	-	OXA-11, OXA-15
2df	D	Carbapenems	V	-	OXA-23, 0XA-48
2e	A	Extended-spectrum cephalosporins	+	-	CEPA
2f	A	Carbapenems	V	-	KPC-2, IMI-1, SME-1
3a (B1)	B	Carbapenems	-	+	IMP-1, VIM-1, IND-1, CcrA
	(B2)				L1, CAU-1, GOB-1, FEZ-1
3b (B3)	B	Carbapenems	-	+	CphA, Sfh-1
4	Unknown	-			

(V), variable, (+), Yes, (-), No, CA, Clavulanic acid, TZB, Tazobactam

1.1.6. The clinically important β-lactamases

In the last fifty years, β-lactamases have attracted much attention owing of their clinical relevance. Actually, they have been admitted to be responsible for a large case number of therapeutic failures. During the early 1960s, TEM-1 was the first plasmid-mediated β-lactamase in Europe and was obtained from *Escherichia coli* (Datta *et al*, 1965). Since then there has been global spread of the TEM-1 genetic structure to other bacterial species (*Pseudomonas aeruginosa, Haemophilus influenza, Neisseria gonorhoeae*) to the extent that it has become the most common resistance gene of all (reviewed by Bradford, 2001). At the same time, the SHV-1 β-lactamase was identified to be encoded by the chromosome of *Klebsiella pneumoniae* (Pitton, 1972) and then subsequently was identified as a plasmid encoded enzyme in *Escherichia coli* (Nugent & Hedges, 1979)

Escherichia coli possessing plasmid encoded TEM-1 and SHV-1 β-lactamases appeared after the introduction of the amidopenicillins such as ampicillin and amoxycillin. Cephalosporins were introduced to overcome this plasmid mediated resistance. The introduction, particularly of the oxyimino-cephalosporins, into the health market, did overcome this resistance for some time. However, mutations started to emerge in TEM-1 and SHV-1 β-lactamases, giving rise to the so-called extended spectrum β-lactamases (ESBLs). The first that was activaely reported was the SHV-2 β-lactamase, detected in a strain *Klebsiella pneumoniae* in Germany (Kliebe *et al*, 1985). These new types of enzymes can destroy third generation β-

lactams (called ESBLs) and are continuously growing particularly in Europe and Asia (Rupp & Fey, 2003; Bradford, 2001; ZEBA, 2005).

1.1.6.1. Class A β-lactamases

Generally, class A β-lactamase enzymes are susceptible to β-lactamase inhibitors such as clavulanate, tazobactam and, to a lesser extent, sulbactam. However the *Klebsiella pneumoniae* carbapenemase KPC may be an exception to this generalization (Papp-Wallace *et al*, 2009). The first plasmid mediated member of this class was described in *Escherichia coli* in 1963, and was named TEM (Datta, & Kontomichalou, 1965). SHV which is another common β-lactamase detected primarily in *Klebsiella pneumoniae* (Matthew *et al*, 1979). TEM and SHV are common β-lactamases described in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*, primarily responsible for urinary tract, bloodstream, and hospital-acquired respiratory tract infections (Buynak, 2006). Although SHV-1 and TEM-1 share about 68% sequence similarity, the active site of SHV-1 is approximately 0.7 to 1.2 Å wider than TEM-1, which gives significant structural implications, particularly associated to the substrate profiles of SHV variants (Tzouvelekis & Bonomo, 1999). *bla*$_{TEM}$ and *bla*$_{SHV}$ may be detected on plasmids but other class A β-lactamases are encoded on the chromosome such as *bla*$_{PenA}$ from *Burkholderia pseudomallei*, or on integrons such *bla*$_{VEB-1}$ in *Pseudomonas aeruginosa* and *Acinobacter baumannii*, and *bla*$_{GES-1}$ from *Klebsiella pneumoniae* (Naas *et al*, 2008).

1.1.6.1.1. Extended spectrum β-lactamases (ESBL) families

ESBLs are primarily found in the Enterobacteriaceae family of Gram negative bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae* (Paterson and Bonomo, 2005). These enzymes are also produced by non-fermentative Gram negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Jacoby & Munoz-Price, 2005). ESBLs are plasmid-mediated enzymes and can hydrolyse oxyimino-cephalosporins, and monobactams but not cephamycins or carbapenems (Bradford, 2001). Also these enzymes, like their parent enzymes, are inhibited *in vitro* by clavulanate (Paterson & Bonomo, 2005). There are several ESBLs genotypes. The most common of these are the SHV, TEM, and CTX-M (Rupp and Fey, 2003). But also there are other clinically important genotypes include VEB, PER, BES-1, BEL-1, SFO-1, TLA, and IBC (reviewed by Jacoby & Munoz-Price, 2005).

According to the Bush and Jacoby scheme (Table 1.1) (Bush & Jacoby, 2010) ESBLs enzymes can be divided into three main groups: group1 cephalosporinases which are not inhibited by clavulanate, group 2 broad spectrum enzymes which comprise the largest group and are generally inhibited by clavulanate except the 2d and 2f groups, and the group3 metallo-β-lactamases. However, most ESBLs are allocated to group 2be which can hydrolyse penicillins, monobactams, and cephalosporins and are inhibited by clavulanate, they are class A according to the Ambler molecular scheme. CTX-M genotype still suits the former criteria for group 1be enzymes (Dhillon & Clark, 2012).

When ESBLs were first identified in the early 1980s, they were shown to be point mutations of the TEM and SHV broad spectrum β-lactamase enzymes, which lead to cephalosporin resistance (Kliebe *et al*, 1985). The mutations in the genes caused these enzymes to have high catalytic activities for β-lactams by lowering the *Km* values (increasing the affinity) for the β-lactams (Knott-Hunziker *et al*, 1982). They have emerged to be a major cause of hospital acquired infections, especially in intensive care unit (Falagas and Karageorgopoulos, 2009). TEM and SHV have been identified worldwide and are described as giving resistance to extended spectrum cephalosporins. The heavy use of such antibiotic has been described as the reason of this resistance (Livermore & Hawkey, 2005). Since the beginning of the 21st century, it has become significantly apparent that a shift in the genotypic structure of ESBLs is taking place (Dhillon & Clark, 2012).

The CTX-M genotype, deriving from chromosomally encoded β-lactamase enzymes of the *Kluyvera* spp, has increased radically, particularly in *Escherichia coli* and *Klebsiella pneumoniae* ((Livermore & Hawkey, 2005). It is thought that the genes were inserted into plasmids then transferred to pathogenic bacteria, with the ability to move on among different bacterial species and genera (Bonnet, 2004). There are more than 50 variants of CTX-M so far, and they have been linked to numerous outbreaks of infections either in hospitals or in the community, especially in urinary *Escherichia coli* isolates in nonhospital settings (Dhillon & Clark, 2012; Falagas and Karageorgopoulos, 2009). Studies from different countries showed that once a CTX-

M β-lactamase penetrates a certain geographic area it becomes predominant with displacement of TEM and ESBL variants (Bonnet, 2004; Livermore and Hawkey, 2005).

1.1.6.1.1.1. TEM β-lactamases

The TEM family of ESBLs represents the largest and widely disributed group among these enzymes. TEM-1 and TEM-2 penicillinases are their evolutionary precursors (Livermore, 1995; Medeiros, 1997; Bradford, 2001). They hydrolyse the β-lactam ring of penicillins, cephalosporins, and related antibiotics and are detected at high rates in hospitals and clinics worldwide (Matagne *et al*, 1998). TEM-1 was the first TEM allele described and isolated from penicillin resistant *E. coli* in 1963 (Datta and Kontomichalou, 1965). The emergence in the 1980s of new cephalosporins such as ceftazidime and cefotaxime onto the market led to growing problems of β-lactamase-producing organisms. This initiated the appearance of modified or new β-lactamases giving resistance to these antibiotics (reviewed by Bradford, 2001).

More than 200 TEM (http://www.lahey.org/Studies/temtable.asp) variants have been identified by now and new genes continue to appear. These TEM variants alter in amino acid sequence by one to five substitutions and many of them alter in resistance phenotype (i.e. the degree of resistance they give to different antibiotics). Although TEM-1 only gives resistance to penicillins and early cephalosporins, the resistance of its derivatives has surpassed second-, third-, and fourth-generation cephalosporins, β-lactamase inhibitors, and monobactams (Merijn *et al*, 2010). TEM-2 was the first

derivative of TEM-1 and had a single amino acid substitution at position 39; however, this did not change the substrate profile although it did change the isoelectric point from 5.4 in TEM-1 to 5.6 in TEM-2. TEM-3 was the first TEM-type β-lactamase that revealed the ESBL phenotype (Sougakoff *et al*, 1988; Payne & Amyes, 1991; Du Bois *et al*, 1995).

The amino acid substitutions that are found within the exteneded-spectrum TEM enzymes concentrate at a limited number of amino acid positions. A few amino acid residues substitutions are particularly significant for producing the ESBL phenotype.The pivotal change is the substitution of arginine164 by either serine or, less commonly, histidine. Arginine 164 forms two ionic bonds with glutamate171 and asparate 179. The substitution of arginine removes those bonds and the small α-7 helix at the entrance of the active site moves to one side allowing the larger cephalosporins to bind and raising the MIC of ceftazidime to around 4mg/L (Du Bois *et al*, 1995).

Following initial substitution at position 164, additional substitutions further increase the MIC of ceftazidime.These changes include glutamate to lysine at position 104 and glutamate to lysine at position 240, which can raise the MIC of ceftazdime to around 64mg/L (Du Bois *et al*, 1995; Perilli *et al*, 1997). A different set of substitutions starting with glycine to serine at position 238, raises the MIC of cefotaxime (Du Bois *et al*, 1995).

TEM-type β-lactamases are most frequently identified in *Escherichia coli* and *Klebsiella pneumoniae*, but they are also described in other species of Gram negative bacteria (Bradford, 2001). TEM-type ESBLs have been identified in non Enterobacteriaceae Gram negative bacteria such as *Pseudomonas aeruginosa* (TEM-42) (Mugnier *et al*, 1996).

1.1.6.1.1.2. SHV β-lactamases

SHV-1 is a narrow spectrum β-lactamases enzyme with activity against penicillins (Matthew *et al*, 1979). This enzyme first identified as a chromosomally encoded β-lactamase in *Klebsiella* species (Nugent & Hedges, 1979). In addition, SHV-1 enzyme is most frequently detected in *Klebsiella pneumoniae* and is responsible for about 20% of plasmid-mediated ampicillin resistance in the latter species (Tzouvelekis & Bonomo, 1999). Several studies showed that the SHV-1 β-lactamase gene is encoded by a transposon and it has been observed on plasmids of different types (Nugent & Hedges, 1979), but the observations of the physical transfer of the bla_{SHV} by transposition has never been proven (Jacoby & Sutton, 1991).

The first emergence of an SHV ESBL was reported in Germany (Knothe *et al*, 1983), which was called SHV-2. There are relatively few SHV-1 variants comparing to TEM-type β-lactamases (http://www.lahey.org/Studies/). The changes that have been seen in bla_{SHV} result in appearance of different SHV derivatives exhibit in fewer positions within the gene. Because of its similarity to SHV-1 the new variant was called SHV-2 (Kliebe *et al*, 1985). A single amino acid substitution changes the spectrum of activity of the SHV-1 β-lactamase to include extended-spectrum

cephalosporins. Glycine at position 238 in SHV-1 is substited by serine in SHV-2 (Barthélémy *et al*, 1988**).** Most of SHV variants expressing an ESBL phenotype are specified by changing of a serine for glycine at position 238, which increases the resistance to cefotaxime. A further substitution at position 240 of lysine for glutamate to give SHV-5 increases the hydrolysis of ceftazidime (Du Bois *et al*, 1995). It is interesting to know that both Gly238Ser and Glu240Lys amino acid changes are similar to those seen in TEM-type ESBLs and perform the same functional changes (Huletsky, Knox, and Levesque. 1993).

Nowadays, most of SHV-type variants express the ESBL phenotype. However, SHV-10 is described to have an inhibitor-resistant phenotype. This variant occurs to be derived from SHV-5 and have one additional amino acid substitution at position 130 of glycine for serine (Prinarakis *et al*, 1997). In addition, most of SHV-type ESBLs are detected in *Klebsiella pneumoniae* (Bradford *et al*, 1997). However, these enzymes have also been detected in strain of *Escherichia* coli (Rasheed *et al*, 1997), *Pseudomonas aeruginosa* (Naas *et al*, 1999), and *Citrobacter diversus* (El Harrif-Heraud *et al*, 1997).

1.1.6.1.1.3. CTX-M β-lactamases

The CTX-M type β-lactamases was identified as a new ESBL family member in 1998 (Bonnet, 2004). The new type did not belong to either the TEM or SHV types though had the characteristics of a class A β-lactamase (Bauernfeind *et al*, 1990). The origin of CTX-M-type ESBLs was completely different from that of TEM- or

SHV-type ESBL (Bonnet, 2004). The CTX-M family of enzymes occurs to have derived from initial transfer of the chromosomal β-lactamase gene from *Kluyvera* spp. to conjugative plasmids that have readily dessiminate among different members of the *Enterobacteriaceae* and other gram-negative bacteria (Canton & Coque, 2006). By the end of the 1990s, the majority of the ESBLs found were either TEM or SHV types which were often related to nosocomial outbreaks caused by *Klebsiella pneumoniae* (Paterson & Bonomo, 2005). The worldwide dissemination of CTX-M-producing *Escherichia coli* has been increasing, and they are now known to be the main ESBL producers and are usually related to community-acquired infections (Pitout & Laupland, 2008).

Unlike other ESBLs types, CTX-M family includes a complex and non-similar group of enzymes. The first analysis and alignment of the amino acid sequences of the CTX-M variants categorised these enzymes into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) (Bonnet, 2004) (Figure 1.5), but recent studies revealed that there are at least two more clusters (Rossolini *et al*, 2008). The phylogenetic analysis of the genes shows that the five main clusters of CTX-M enzymes exhibit >94% identity with other members of the same group, while there was ≤90% between members of different groups (Bonnet, 2004). The CTX-M-1 group contains six plasmid-mediated enzymes CTX-M-1(Barthe´le´my *et al*, 1992), CTX-M-3 (Gniadkowski *et al*, 1998), CTX-M-10 (Karim *et al*, 2001), CTX-M-12 (Kariuki *et al*, 2001), CTX-M-15 (Matsumoto *et al*, 1988), and FEC-1 (Oliver *et al*, 2001)). The CTX-M-2 group contains eight plasmid-mediated CTX-M enzymes (CTX-M-2 (Bauernfeind *et al*, 1996), CTX-M-4, CTX-M-4L (Gazouli *et al*, 1998),

CTXM-5 (Bradford *et al*, 1998), CTX-M-6 (Gazouli *et al*, 1998), CTX-M-7 (Ishii *et al*, 1995), CTX-M-20 (Saladin *et al*, 2002), and Toho-1 (Tassios *et al*, 1999). The CTX-M-8 group contains one plasmid-mediated member (Bonnet *et al*, 2000). The CTX-M-9 group contains nine plasmid-mediated enzymes (CTX-M-9 (Sabate´ *et al*, 2000), CTX-M-13, CTX-M-14 (Chanawong *et al*, 2002), CTX-M-16 (Bonnet *et al*, 2001), CTX-M-17 (Cao *et al*, 2002), CTX-M-19, CTX-M-21, CTX-M-27 (Bonnet *et al*, 2003), and Toho-2 (Labia, 1999). To date, CTX-M variants exceed 135 enzymes (http://www.lahey.org/Studies). Phylogenetic analysis indicated that CTX-M β-lactamases emerged not by mutations from earlier plasmid mediated enzymes but by mobilization of chromosomal *bla* genes from *Kluyvera* species. These bacteria are closely related to *Escherichia coli* and found worldwide (Canton and Coque, 2006). The *bla* genes were integrated into mobile genetic structures and transferred presumably by conjugation into clinical bacteria (Canton *et al*, 2008). These mobilized *bla*$_{\text{CTX-M}}$ genes increase cefotaxime resistance to a much greater degree than resistance to ceftazidime.

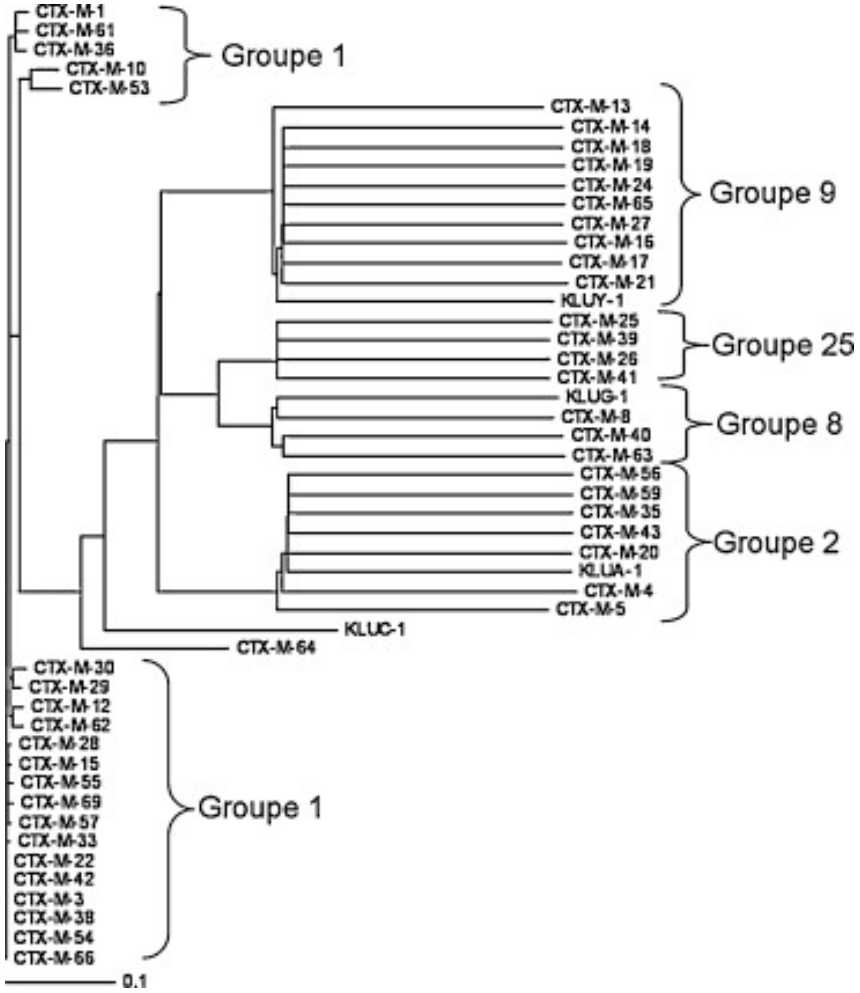


Figure 1.5: Phylogeny tree of CTX-M family show every group of CTX-M with its types accordingly (adapted from Ruppé, 2010).

The reasons why CTX-M enzymes have had the opportunity to increase the hydrolytic activity against ceftazidime in the new variants are probably because they have (i) diverge by mutations as consequence of antibiotic selective pressure on *Kluyvera* species. (ii) incorporated of $bla_{\text{CTX-M}}$ genes into mobile genetic elements (Poirel *et al*, 2008).

Reports over the last 10 years revealed that with some exceptions, the CTX-M have nearly replaced other ESBLs enzymes in Enterobacteriaceae, involving TEM and SHV ESBL variants (Cantón, 2008; Coque *et al*, 2008). This replacement might have appeared not only as a result of the extraordinary spread of the corresponding *bla*$_{\text{CTX-M}}$ genes in mobile genetic structures, including transposons and plasmids, but also because of the presence of these structures within successful clones (Cantón & Coque; Rogers *et al*, 2011). Another reason for this rise may be the co-resistancephenomenon in CTX-M producing isolates, especially to aminoglycosides and fluoruquinolones, which can facilitate co-selection process (Cantón and Ruiz-Garbajosa, 2011).

The presence of co-resistance gives advantages to the bacteria under selective forces of antibiotics. It is known that the emergence of antibiotic resistance is a direct result of the consumption of the antibiotics and that resistance is appeared by mutational events in a Darwinian way (Sykes, 2010). However, selection does not only include mutations but also resistance genes transferred by horizontal transfer (Canton & Morosini, 2011). The pre-existence of resistance genes in the nature that might be captured and inserted in the bacterial genome resulting in resistance phenotypes is widely known (Canton, 2009).

The significance of plasmids in the dissemination of *bla*$_{\text{CTX-M}}$ genes can be seen with specific CTX-M β-lactamases and has been reported in several studies (Carattoli, 2009). For example, *bla*$_{\text{CTX-M-15}}$ genes are mostly associated with plasmids of incompatibility group FII (Coque *et al*, 2008). They are characterized by own copy

number in the bacteria. These genes are also detected in Enterobacteriaceae and have previously been named as epidemic resistance plasmids because of their ability to acquire and transfer resistance genes among bacteria (Carattoli, 2009). Within the FII plasmids, different replicon types may be present that could facilitate quick evolution and plasmid diversification. In addition, studies on IncFII plasmids proposed that they were excessively disseminated within Enterobacteriaceae, even before antibiotic use, and fully adapted to these bacteria (Datta *et al*, 1980). This may have facilitated the existence and dissemination of resistance genes after acquisition, including *bla*$_{\text{CTX-M}}$ genes. Furthermore, linkage of these genetic determinants to genes mediating non-β-lactam resistance mechanisms might have also provided the key to the maintenance of these plasmids within the organisms and thus the *bla*$_{\text{CTX-M}}$ genes under co-selection process (Woodford *et al*, 2009).

Other broad host range replicon plasmids like IncN, IncI1, and IncL/M have also been included in the spread and dissemination of CTX-M enzymes. Some of these plasmids, such as IncN, could have their reservoir in animals and have been isolated extensively in *Escherichia coli* and *Salmonella* species isolated from food producing animals (Carattoli, 2011). These plasmid usually carry *qnr* or *aac(6)-Ibcr* genes giving low level fluoroquinolone resistance (Bado *et al*, 2010). Recently, these plasmids have been shown to be able to harbour genes coding the emerging carbapenemase like *bla*$_{\text{NDM-1}}$ (Poirel *et al*, 2011) and have been described as epidemic plasmids in different countries with a predominance of carbapenem resistance (Schwaber & Carmeli, 2008).

The IncL/M group of plasmids have the ability to carry *bla*$_{CTX-M}$ genes, such as *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-15}$ (Sabtcheva *et al*, 2008). Other types of plasmids such as IncK plasmid have been associated with *bla*$_{CTX-M-14}$ in southern Europe (Cottel *et al*, 2011), this incompatibility group has also been associated with the dissemination of *bla*$_{CMY}$ genes (Baudry *et al*, 2009). The IncH12 plasmids have been associated with the spread of the *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-9}$ in other geographical regions (García-Fernández *et al*, 2007) and it has also been linked with the dissemination of *bla*$_{VIM}$ and qnr genes (Oteo *et al*, 2010b) These studies indicate the ability of individual plasmid groups to recruit resistance genes, thus enhancing the persistence and prevalence of these resistance genes in bacteria. Moreover, it is proposed that persistence for along time also provides the opportunity to create gene variation and thus an increasing number of *bla*$_{CTX-M}$ variants to deal with all cephapsporin types. This is similar to what has happened with the OXA-type β-lactamases (Evans, Hamouda & Amyes, 2007). Some of these *bla*$_{CTX-M}$ variants have been recovered in *vitro* under selective pressure of antibiotics but have not yet been detected in clinical isolates (Ripoll *et al*, 2011).

One of the important issues in the dissemination of CTX-M enzymes is the participation of certain clones of inidivdual bacterial species, especially those from *Escherichia coli* and *Klebsiella pneumoniae*. In addition, it has suggested that despite a high diversity of CTX-M producers, a few clones or sequence types clustered in clonal complexes have been frequently associated with CTX-M enzymes. This

denotes that these clones are involved in the spread of these enzymes and that the success of specific CTX-M enzymes might also rely on certain sequence types or clonal complexes where they are repeatedly exist (Woodford *et al*, 2011). The presence of high risk clones in the nature increases the chance of accumulating resistance genes. In Gram negative bacteria, the ST131 *E. coli* was identified worldwide as carrying the *bla*CTX-M-15 gene, which encodes the successful ESBL (Cantón & Ruiz-Garbajosa, 2011).

A connection between *bla*CTX-M genes and certain surrounding genetic components, including Insertion sequences, integrons, and transposons has been identified. These genetic elements are incorporated into more complex structures; for example different plasmid replicon types and certain clones giving a complex hierarchical organization with the opportunity of exchanging different modules (Carattoli, 2011; Woodford *et al*, 2011). *bla*CTX-M genes have been theoretically mobilized from *Kluyvera* species by insertion sequences or by lesser extent bacteriophages. The insertion sequence plays a role in the over-expression of *bla*CTX-M genes and some of these such as IS*CR1* are joined to integron structures that are also joined to transposition units (Baquero, 2004; Cantón & Ruiz-Garbajosa, 2011). In addition, these structures are usually integrated within conjugative plasmids that might be included in successful clones. Their presence in the successful clones has been suggested to be crucial to the evolutionary success of the bacteiral clone (Baquero, 2004; Canton and Ruiz-Garbajosa, 2011). Some of these structures and plasmids, which harbour *bla*CTX-M genes, also carry other resistance genes, including genes encoding carabapenemases and AmpC β-lactamases (plasmid *bla*AmpC), methylases

affecting aminoglycosides, or plasmid-mediated quinolone resistance genes (*qnr* genes). All these genes could also confer an advantage for the maintenance of *bla*$_{CTX-M}$ genes because of co-selection pressure (Cantón *et al*, 2012).

There are several studies identifying new or unique genetic rearrangement linked to *bla*$_{CTX-M}$ genes but further analysis showed that these are only variations of a small number of different genetic structures (Lartique *et al*, 2004, Eckert *et al*, 2006, Dhanji *et al*, 2011). The mobilization of *bla*$_{CTX-Ms}$ genes has been by insertion sequences placed astride the gene. Furthermore, in the insertion sequence upstream of the gene, there is often a promoter enhancing *bla*$_{CTX-M}$ expression. So the insertion sequence upstream of *bla*$_{CTX-Ms}$ genes has a role both in the selection and spread of these genes. The downstream sequences of *bla*$_{CTX-M}$ genes are more variable and there is less information about them. (Cantón *et al*, 2012).

Many different insertion sequences have been described upstream of *bla*$_{CTX-Ms}$ genes, including IS*Ecp*1, IS*CR*1, IS*10*, and IS*26* (Eckert *et al*, 2006; Bae *et al*, 2008). IS*Ecp*1 is the most frequently insertion sequence detected upstream of different *bla*$_{CTX-M}$ genes. Although it was first reported linked to the *bla*$_{CTX-M-15}$ gene in 1999, it has been associated with all groups of these β-lactamases except *bla*$_{CTX-M-8}$. In addition, IS*10* has been associated with *bla*$_{CTX-M-8}$ and IS*CR1* with several members of *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-9}$ clusters. The number of new genetic environments upstream of *bla*$_{CTX-M}$ can be associated with the number of mobilizations. A new insertion sequence can interupt an insertion sequence already present upstream of

*bla*CTX-M; for example, IS*1*, IS*10*, IS*CR1*, and IS*26* have been shown to be capable of inserting into IS*Ecp*1 (Eckert *et al*, 2006; Bae *et al*, 2008). To date, there have been descriptions of at least nine mobilization situations: three in CTX-M-1 cluster (*bla*CTX-M-10, *bla*CTX-M-53, and other common *bla*CTX-M genes included in this group), two in CTX-M-2cluster (*bla*CTX-M-2 and *bla*CTX-M-5), one in CTX-M-8 and CTX-M-25 (*bla*CTX-M-8 and *bla*CTX-M-25, respectively), and two in CTX-M-9 cluster (*bla*CTX-M-9 and *bla*CTX-M-14) (Cantón *et al*, 2012).

Spacer sequences between the genes of IS*Ecp*1 and *bla*CTX-M have been well described. The genetic distance between insertion sequence and *bla*CTX-M gene has been associated with the level cephalosporin MIC values (Ma *et al*, 2011). These genetic distances range from 48 to 127 bp in the *bla*CTX-M-1 gene cluster, about 34 to 42 bp in *bla*CTX-M-9 gene cluster and around 40 to 52 bp in *bla*CTX-M-25 and *bla*CTX-M-8 gene clusters respectively. When the similarity among spacer sequences of *bla*CTX-M-1 gene cluster was examined, most contained a common region with some minor exceptions such as those found with *bla*CTX-M-10 and *bla*CTX-M-53. This denotes that the *bla*CTX-M genes belonging to the *bla*CTX-M-1 cluster might have originated from a single transposition event. In these clusters, the source was *Kluyvera georgiana*. However, the absence of homology in spacer sequences of these *bla*CTX-M gene clusters denotes a different source for each one. These results demonstrate that a *Kluyvera* species, which has not been described so far, might be the source of clusters currently attributed to *Kluyvera georgiana* (Cantón *et al*, 2012).

It is admitted that chromosomal *bla*$_{Klu}$ genes are poorly expressed in the original strain and need the presence of a strong promoter upstream to enhance the MIC values, leading to phenotypic resistant isolates Although the capture of IS*Ecp*1 upstream of chromosomal *bla*$_{klu/CTX-M}$ genes appears at a high rate in laboratory settings, these insertion sequences have not been identified in natural isolates of *Kluyvera* (Lartigue *et al*, 2006). When IS*Ecp*1 was integrated upstream of *bla*$_{Klu/CTX-M}$ gene, a stress situation, such as that imposed by the antibiotics cefotaxime or ceftazidime, enhances the mobilization of IS*Ecp*1 and the transfer of the *bla*$_{Klu/CTX-M}$ gene (Lartigue *et al*, 2006; Nordmann *et al*, 2008).

1.1.6.1.1.4. Other ESBLs enzymes

Whereas the majority of ESBLs are coming from TEM or SHV β-lactamases and others might be classified with one of the newer families of ESBLs, several ESBLs have been described that are not closely related to the well-known families of β-lactamases (reviewed by Bradford, 2001).

PER-1 was first reported in *Pseudomonas aeruginosa* (Nordmann *et al*, 1993), and later found in *Salmonella enterica* (Gierczynski *et al*, 2003), *Klebsiella pneumoniae* (Paterson *et al*, 2003), *Acinetobacter baumannii* (Yong *et al*, 2003) and *Proteus mirabilis* (Pagani *et al*, 2002). PER-2 has been found in *Salmonella enterica* (Bradford, 2001), *Enterobacter* species (Quinteros *et al*, 2003), *Klebsiella pneumoniae* (Melano *et al*, 2003), and *Vibrio cholerae* (Petroni *et al*, 2002). There

have subsequently been a number of new PER enzymes found (Opazo *et al*, 2012). VEB-1 has been detected in *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* (Cao *et al*, 2002), *Pseudomonas aeruginosa* (Chanawong *et al*, 2001), and *Enterobacter* species (Girlich *et al*, 2001). All of these enzymes preferentially hydrolyse and enhance resistance to both ceftazidime and aztreonam (Poole, 2004). The CME-1 and TLA-21 ESBLs found in *Chryseobacterium meningospeticum* and *Escherichia coli*, respectively (reviewed by Bradford, 2001). Other uncommon ESBL enzymes include the BES-1 enzyme found in *Salmonella marcescens* (Bonnet *et al*, 2000), the SFO-1 enzyme in *Enterobacter cloacae* (Matsumoto & Inoue, 1999), The FEC-1 enzyme observed in *Escherichia coli* (reviewed by Bradford, 2001), the IBC-1 enzyme detected in *Enterobacter cloacae* (Giakkoupi *et al*, 2000), the GES-1 enzyme found in *Klebsiella pneumonia* (Correia *et al*, 2003).

1.1.6.1.2. Class A carbapenemases

Serine carbapenemases class A β-lactamases, of functional group 2f, have occurred infrequently since their first detection in 1986 (Medeiros & Hare, 1986). These β-lactamases have been described in members of the Enterobacteriaceae, especially *Klebsiella pneumoniae*, and *Serratia marcescens* in small outbreaks and in single isolates (Medeiros & Hare, 1986; Nordmann, 1993; Yang, Wu & Livermore, 1990). Isolates exhibiting these enzymes are known by reduced imipenem susceptibility, but MICs range from intermediate (i.e. imipenem MICs of ≤4 μg/ml) to fully resistant. Therefore, carbapenemase enzymes might be difficult to recognise when using routine susceptibility testing (Queenan & Bush, 2007).

Three main families of class A serine carbapenemases have been described, which include KPC, NMC/IMI, and SME enzymes. Their hydrolytic activity requires an active site serine at position 70 (Ambler *et al*, 1991). All these enzymes have the ability to hydrolyze a wide variety of β-lactams, including penicillin, cephalosporins, aztreonam as well as carbapenems, however all are inhibited by tazobactam and clavulanate, locating them in the group 2f functional subgroup of β-lactamases. The GES β-lactamases, which was classified as ESBL family, but then variants were detected that had low measurable imipenem hydrolysis. Consequently, this subgroup of GES enzyme is also categorized as functional group 2f carbapenemases (Queenan and Bush, 2007).

The major chromosomal encoded serine class carbapenemase enzymes are SME, NMC, and IMI. These enzymes are classified in group 2f β-lactamases according to Amber classification (Queenan and Bush, 2007). SME-1 enzyme was first found in England from *Serratia marcescens* in 1982 (Nass *et al*, 1994). Infections caused by SME-producing *Serratia marcescens* were detected as single cases or small clusters. The IMI and NMC-A β-lactamases have been found in rarely in clinical isolates of *Enterobacter cloacae* in France (Rasmussen and Bush, 1996), United States (Pottumarthy *et al*, 2003) and Argentina (Radice *et al*, 2004). The NMC-A and IMI-1 β-lactamases have 97% amino acids similarity and are related to SME-1, with nearly 70% amino acid similarity (Nass *et al*, 1994; Rasmussen and Bush, 1996). These β-lactamases genes are all located chromosomally, with no evidence of location within

mobile structures (Aubron *et al*, 2005). These enzymes hydrolyse imipenem more efficiently than meropenem, and show poor hydrolysis of cefoxitin and extended spectrum cephalosporins (Mariotte-Boyer *et al*, 1996; Queenan *et al*, 2000; Rasmussen and Bush, 1996).

These chromosomal carbapenemase β-lactamases might be induced in response to challenge by cefoxitin and imipenem. Sequencing of the upstream regions of NMC-A, IMI-1, and SME-1 identified the existence of a divergently transcribed gene linked to the LysR family of DNA binding transcriptional regulatory genes (Naas and Nordmann, 1994; Nass *et al*, 1994; Rasmussen and Bush, 1996). Deletions within the *nmcR* gene stopped the inducibility of NMC-A and decreased carbapenem MICs, confirming a role for this protein to enhance NMC-A expression (Naas and Nordmann, 1994).

Two structures of NMC-A and a structure for SME-1 reveal that all structures and catalytic residues are similar to other class A β-lactamases (Mourey *et al*, 1998; Sougakoff *et al*, 2002; Swaren *et al*, 1998). In the NMC-A β-lactamases, the structure of the enzyme did not alter when the inhibitor was bound. In fact, the disulphide bond between position 69 and 238 which is located near the active site, is essential for hydrolytic activity, not only for imipenem hydrolysis, but also for the stabilization of the enzyme structure (Majiduddin, and Palzkill, 2003; Sougakoff *et al*, 2002). In the case of the SME-1 β-lactamases, the hydrolysis of imipenem could not be atrributed to individual residues of the SME-1 active site alone, multiple

residues influence the active site structure and therefore affect substrate specificity (Majiduddin & Palzkill, 2005).

On the other hand, there are transferable plasmid-encoded class A carbapenem-hydrolysing β-lactamases, which include KPC (for *Klebsiella pneumoniae* carbapenemase) and GES. The spectrum of hydrolysis of the KPC β-lactamases includes third generation cephalosporins (such as cefotaxime) as well as carbapenems. These β-lactamases are most frequently detected in *Klebsiella pneumoniae* but also have been identified in *Enterobacter* and *Salmonella* species (Bratu *et al*, 2005b; Hossain *et al*, 2004; Miriagou *et al*, 2003).

The first member of the enzyme KPC family (KPC-1) was detected in a clinical isolate of *Klebsiella pneumoniae* from the United States in 1996 (Yigit *et al*, 2001). This isolate was resistant to all β-lactam drugs. The detection of KPC-1 was followed by the discovery of KPC-2 in 2003, resulting from a point mutation in KPC-1 (Moland *et al*, 2003). The *bla*$_{KPC-2}$-producing gene was also carried on a transferable plasmid. Both enzymes showed reduced susceptibility to imipenem (Moland *et al*, 2003). Another single amino acid variant of KPC-2 produced KPC-3 which was identified in a strain isolated in 2000 in the *Klebsiella pneumoniae* outbreak in New York (Woodford *et al*, 2004). Analysis of the KPC-3 enzyme showed a profile similar to that of KPC-1 and KPC-2, with slight rise in ceftazidime hydrolysis (Alba *et al*, 2005).

KPC class of carbapenemases have undergone rapid spread from the United States e. Since the detection of KPC-2, it was quickly found in Colombia (Villegas *et al*, 2006) and China (Wei *et al*, 2007). The of KPC-2 gene on a plasmid in *Pseudomonas aeruginosa* shows a disturbing development in the dissemination of these carbapenemases (Villegas *et al*, 2007).

The conserved active sites of the KPC enzymes class A carbapenemases have amino acid identity of around 45% to the SME carbapenemases. These enzymes have conserved cysteine residues at positions 69 and 238 that form a similar disulphide bond to those in the SME and NMC/IMI enzymes. The KPC-2 β-lactamase structure shows characteristic conserved motifs, when compared to the carbapenemases such as SME-1 and NMC-A; and non-carbapenemases such as TEM-1 and SHV-1 (Ke *et al*, 2007).

Among the functional group 2f carbapenemase enzymes, the KPC family has been more frequent to disseminate owing to its location on transferable plasmids, as it is most commonly detected in *Klebsiella pneumoniae*. It is extremely difficult to treat infections caused by this organism because of its propensity to be multidrug resistant (Bratu *et al*, 2005).

1.1.6.1.3. Class C AmpC-type β-lactamases

Class C β-lactamases (AmpC) are an important contributor to multiple resistance of β-lactam antibiotics in Gram-negative bacteria, particularly the Enterobacteriaceae (Goossens, 2001; Garcia-Rodriguez and Jones, 2002). These enzymes were originally the chromsomal mediated enzymes of Gram-negative bacteria; however, the *bla*$_{AmpC}$ migrated onto plasmids from strains such as *Citrobacter freundii* (Woodford *et al*, 1990; Payne *et al*, 1992, Fosberry *et al*, 1994) and thus could be transported into *Esherichia coli*. They show activity against penicillins but are more active against oxyiminocephalosporins (cefotaxime, ceftazidime, and ceftriaxone). They can also hydrolyse cephamycins (cefoxitin and cefotetan) and monobactams (aztreonam), but are susceptible to carbapenems (and fourth generation cephalosporins (cefepime and cefprome) (Coudron *et al*, 2003). According to the Ambler structural classification, AmpC β-lactamases belong to class C, according to the functional classification they were placed in group 1 (Bush *et al*, 1995).

Genes encoding enzymes of AmpC are located on the chromosomes of some Enterobacteriaceae (Walther-Rasmussen, and Hoiby, 2002; Stock *et al*, 2003; Stock and Wiedemann, 2003) and other Gram negative bacteria such as *Pseudomonas aeruginosa* (Walther-Rasmussen, and Hoiby, 2002), and *Acinobacter baumannii* (Nadjar *et al*, 2001). With the exception of *Escherichia coli*, these enzymes are inducible by some of β-lactam antibiotics. Although carbapenems are good inducers of these enzymes, their stability and prompt bactericidal activity to hydrolysis however, makes them effective against AmpC-producing bacteria (Jones, 1998).

Chromosomal *amp*C genes expression is typically effected by the AmpR regulator and the *ampD*-encoded amidase which act negatively on *ampC* expression (Hanson and Sanders, 1999). AmpC-mediated resistance typically appears from mutational *ampC* gene expression, usually due to mutations in *ampR* and or *ampD* (Hanson and Sanders, 1999; Petrosino *et al*, 2002; Bagge *et al*, 2002). Recent studies on AmpC-mediated β-lactam resistance reveal that ampC overexpression might appear as a result of upstream insertion of insertion sequences (Corvec *et al*, 2003; Segal *et al*, 2004).

Plasmid-encoded AmpC β-lactamases have now become increasing significant as as a mechanism of resistance to expanded-spectrum β-lactams. *Klebsiella pneumoniae* (Steward *et al*, 2001) and *Escherichia coli* (Philippon *et al*, 2002) often harbour these enzymes. Most plasmids harbouring *ampC* genes are not inducible due to the lack of *ampR* regulatiry gene. The overproduction of the plasmid *bla*$_{\text{AmpC}}$ is under the control of plasmid-encoded promoter in resistant bacteria (Reisbig *et al*, 2003). Many of the plasmid-borne *bla*$_{\text{AmpC}}$ genes are associated with integrons and transposable structures, so unlike plasmid-borne β-lactamase enzymes of classes A, B, and D, they are not included in characteristic gene cassettes. AmpC plasmids might harbour additional resistance genes for non-β-lactam antibiotics (Rodriguez-Martinez *et al*, 2003; Zhao *et al*, 2003). Plasmid-borne AmpC β-lactamases are classified into five families according to their amino acid sequence (Rodriguez-Martinez *et al*, 2003).

1.1.6.1.4. Class D OXA-type- β-lactamases

The OXA-type β-lactamases belong to class D group according to Ambler classification, and are frequently found in *Pseudomonas aruginoasa*, but also found less frequently in some Gram negative organisms such as *Acinetobacter baumannii* (Nass & Nordmann, 1999; Paterson & Bonomo, 2005; Pournaras *et al*, 2006). Currently, over 18 OXA extended spectrum β-lactamases have been described (http://www.lahey.org/Studies/). Initially, OXA β-lactamases were known for their high degree hydrolysis of oxacillin and cloxacillin, however most of them did not affect the carbapenem and extended spectrum cephalosporins (Nass and Nordmann, 1999). OXA-45 variant gives resistance to the third generation cephalosporins, aztreonam, and a fourth generation cephalosporins, cefepime (Ibuka *et al*, 2003). Their existence in plasmids and transposons may ease their horizontal movement, although some of them have been located on chromosomes (Nass and Nordmann, 1999; Walther-Rasmussen & Hoiby, 2006). OXA-type ESBLs typically harbour multiple mutations; for example, substitutions at Gly167 are responsible for resistance against ceftazidime in OXA-10-derived ESBLs (Majiduddin *et al*, 2002).

The OXA β-lactamases are the chromosomal β-lactamases of *Acinetobacter* spp, which is probably the source of these genes. Because of their predisposition to cause carbapenem resistance the OXA β-lactamases are also the biggest source of carbapenemases in *Acinetobacter baumannii* (reviewed by Evans, Hamouda & Amyes, 2013).

1.2. Aims

1. To detect the spread of CTX-M-β-lactamases in clinical isolates of bacteria in St George's Hospital London from where the strains were collected.
2. To detect the resistance profiles of the collected isolates and determine MDR patterns.
3. To detect the genotypic relatedness using PFGE among the collected isolates and possible spread of a certain clone.
4. To detect the most common member of CTX-M family of enzymes.
5. To provide analysis of the genetic environment of $bla_{\text{CTX-M}}$ genes.

Section B

Chapter -2: Materials and Methods

2.1. Sample collection and storage

One hundred isolates of mixed species of Enterobacteriaceae were received from St George's London hospital in March 2010. All isolates were inoculated onto MacConky agar (Oxoid) and then incubated 24h at 37^0C. A single colony was added to the beads of Cryobank Sysytem (Mast Diagnostic, UK) to each vial for long term storage at -70^0C following the manufacturer's instructions. The isolates in this study included 50 *Escherichia coli*, 12 *Klebsiella pneuomoniae*, 5 *Klebsiella oxytoca*, 7 *Enterobacter cloacae*, one *Enterobacter aerogens*, one *Enterobacter* spp., 12 *Proteus mirabilis*, one *Proteus vulgaris*, six Lactose fermenting coliform, two isolates *Pantoea* spp., one *Morganella morgana*, one isolate *Hafnia alvei*, and one *Serratia marcescens* as listed in Table 2.1. The source of all isolates in this study was from blood cultures.

Table 2.1: Total number of each Enterobacteriaceae species.

Isolate species	**Number of isolates**
Escherichia coli	50
Klebsiella spp	17
Enterobacter spp	9
Proteus spp	13
Lactose Fermenting Coliform	6
Pantoea spp	2
Serratia marcescens	1
Morganella morganii	1
Hafnia alvei	1
Total	100

2.2. Chemicals, buffers, and media

All chemicals used in this study were bought from Sigma-Aldrich Company (Poole, UK) Ltd unless otherwise stated. Also, media were purchased as powder from Oxoid (Basingstoke, UK) and then preparation was done with distilled water before

sterilization process. Normal saline was made with 0.85% NaCl and sterilized before use.

2.3. Determination of minimum inhibitory concentrations (MICs)

The isolates in this study were examined for their susceptibility to four different classes of antibiotics to observe their spectrum activity. The antibiotics, their classes and the breakpoints used in this study are listed in Table 2.2. The minimum inhibitory concentrations (MICs) were determined by double dilutions agar method according to the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2010). The breakpoints of these antibiotics were described by Andrews (2012). The control strains used in this study were *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Staphylococcus aureus* (*S. aureus*) NCTC 6571.

Table 2.2: Antimicrobial agents name, class, breakpoint and manufacturer.

Name of the antimicrobial agent	Class of the antimicrobial agents	Antimicrobial agent breakpoint	Manufacturer
Cefotaxime	Third generation cephalosporin	2 mg/L	Sigma-Aldrich
Cefoxitin	Second generation cephalosporin	8 mg/L	Sigma-Aldrich
Ceftazidime	Third generation cephalosporin	4 mg/L	Sigma-Aldrich
Ciprofloxacin	second generation flouroquinolone	1 mg/L	(Bayer AG, Germany)
Gentamicin	Aminoglycoside	4 mg/L	Sigma-Aldrich
Imipenem	Carbapenem	8 mg/L	(Merck Sharpe Dohme, Rahway, NJ, USA)
Meropenem	Carbapenem	8 mg/L	(ASTRA Zeneca, Loughborough, UK)
Ertapenem	Carbapenem	1 mg/L	(Merck Sharpe Dohme, Rahway, NJ, USA)

2.4. Double disc synergy method

Synergy between cefotaxime and clavulanate was detected by placing a disc of amoxicillin + clavulanate 20 + 10µg; up, cefotaxime 30µg; and down, ceftazidime 30µg . A clear-cut extension of the edge of the cefotaxime and ceftazidime inhibition zone toward the disc containing clavulanate (as shown in figure 3.1) was interpreted as synergy. The double-disc synergy test was considered positive when decreased susceptibility to cefotaxime was combined with synergy between ceftazidime and clavulanate.

2.5. 10X TAE buffer

10X TAE was prepared by the following procedure (Biokeystone Co LLC, California, USA): 48.44g of Trizma base (Sigma), 3.72g of EDTA (Ethylenediaminetetraacetic acid) disodium salt dehydrate 99%, 11.4 ml glacial acetic acid. It was mixed well until each component had dissolved completely and the pH was adjusted to 8.0. The solution was made upt to a final volume of 1L with distilled water. It was diluted 1:10 ready for use.

2.6. PCR reagents

PCR reagent were prepared from Promega reagents (Promega, Southampton, UK), Go Taq DNA Polymerase (5u/µl). In 5x colourless or green buffer, $MgCl_2$ solution (25mM) and deoxynucleoside triphosphates (dNTP) / PCR nucleotide mix (10mM each). Sterilized distilled water was used as the diluents.

2.7. PCR reaction

All PCR reactions, unless otherwise, stated were performed in 50 µl volumes in PCR tubes. The reaction mixture consisted of 1.5mM (2 µl) $MgCl_2$, 0.1mM (1 µl) dNTPs, 1X reaction buffer (10 µl), 0.05 mM (0.5 µl) each primer, 1 µl template and 0.2 units of *Taq* in sterilized distilled water.

2.8. Screening of PCR products

Agarose (1g) was added to 100 ml TAE buffer (40 mM Tris acetate, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The agarose was solubilized by heating in a microwave oven for about 10 minutes and then the mixture was left to cool to room temperature. Then, five µl of each PCR product samples were put in the gel alongside 5 µl of a suitable molecular weight marker; in each case this was applied after mixing with 1 µl loading buffer on a piece of parafilm. Each mixture was applied to a slot prepared in the gel using 10 µl micropipette. The electrophoresis gel was covered and the power supply was switched on and adjusted to 10 Volt/cm. For staining the gel was immersed in 3X staining solution of GelRed dye (Biotium, Hayward, USA). GelRed was prepared from a stock reagent of the 3X staining solution which was prepared with 45 ml sterile distilled water, 5 ml of (1 M) NaCl, and 15 µl of the stock solution and left at room temperature for storage and further use. After running, the gel was visualized in an Ultra Violet transilluminator Bio-Rad Gel Doc 2000 (Bio-Rad, Hemel Hempstead, UK).

2.9. Purification of PCR product

PCR products were purified either directly or by separation of the DNA fragments from 1%TAE Agarose gel electrophoresis. The DNA band on the gel of the correct size was sliced out from the gel. The purification of DNA by both methods was performed using a Qiagen purification kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

2.10. Determination of DNA concentration

The concentration of the purified DNA and its purity was measured by UV absorbance at 260 and 280 nm. The concentration of DNA was determined by the OD260nm (1 OD_{260nm} = 50 µg/ml dsDNA or 33 µg/ml ssDNA). The purity was calculated with the OD260nm/OD280nm ratio, with a ratio of ca. 1.8 indicating a low degree of protein contamination.

2.11. Sequencing of genes

The resulting PCR products were sequenced by the Sanger method using QIAquick PCR Purification Kit (Qiagen, Crawley, UK) and sequenced by ABI3730 capillary sequencer (Applied Biosystems, Warrington, UK). The DNA sequencing results were analysed by using BioEdit, Multalin, EXPASY, Basic Local Alignment Search Tool (BLAST). The results of sequencing were compared with the previous published genes sequences in an online website (http://www.ncbi.nih.gov/BLAST) and (http://www.lahey.org/studies/webt.stm). The sequencing alignment results and protein sequences were achieved by the Multialin website (http://www.toulouse.inra.fr/multalin.html).

2.12. Phenotypic and molecular identification of ESBLs genes

For ESBL screening, the strains in this study were identified phenotypically with agar double dilution method. Then, confirmation of ESBL-production was done by disc diffusion. The concept of this test is to examine the presence of ESBL enzymes (Jarlier *et al*, 1988). According to the British Society for Antimicrobial Chemotherapy (BSAC) disc susceptibility test, one disc containing co-amoxiclav 20+10 µg is placed in the centre of an Isosensitest (ISO) agar plate between ceftazidime 30 µg and cefotaxime 30 µg discs. The former two discs are applied 25-30mm a part (Livermore & Williams, 1996). The plate was incubated overnight at 37° C, and numbers of selected CTX-M producers are examined.

2.13 CTX-M gene detection

Multiplex PCR was performed for identification of CTX-M genes using specific primers designed for identifying known β-lactamase genes (Individually, CTX-M groups 1, 2, 9, and, together, groups 8 and 25). Multiplex PCR templates were prepared by boiling a dense bacterial suspension in sterile distilled water; one µl of the boiled cell suspension supernatant was used in the PCR reaction. Primer pairs and predicted amplicon sizes are listed in Table 2.3. Whereas, frequents of the genes of group 8 (666bp) and 25 (327bp) were amplified with two specific forward primers and a shared reverse primers (Table 2.3) The latter two groups were developed and optimized empirically (Woodford *et al*, 2006).

PCR amplifications were performed using *Taq* polymerase (Promega) (initial denaturation at 94°C for 5 min., 30 cycles of 94°C for 25s, 52°C for 40s and 72°C for 50s, and a final elongation at 72°C for 6 minutes). In addition, the resulting PCR products were analyzed in 1.5% agarose gel, using the following electrophoresis: 30 minutes, 100V, TAE buffer 1X.Both ethidium bromide and GelRed staining were used for visualization amplified products under an UV light (Woodford *et al*, 2006). The preparation of 1X TAE buffer was done by diluting a stock 10XTAE buffer 1:10.

Table 2.3: Primers used for the detection of *bla*$_{CTX-M}$ genes.

Name	**Primer**	**Amplicon size (bp)**
CTX-M-1 group members	F-5'-AAA AAT CAC TGC GCC AGT TC R-5'-AGC TTA TTC ATC GCC ACG TT	415
CTX-M-2 group members	F-5'-CGA CGC TAC CCC TGC TAT T R-5'-CCA GCG TCA GAT TTT TCA GG	552
CTX-M-9 group members	F-5'-CAA AGA GAG TGC AAC GGA R-TG 5'-ATT GGA GGT TCA TCA CC	205
CTX-M-8 group members	F- 5'-TCG CGT TAA GCG GAT GATGC R-5'-AAC CCA CGA TGT GGG TAG C	666
CTX-M-25 group members	F-5'-GCA CGA TGA CAT TCG GG R-5'-AAC CCA CGA TGT GGG TAG C	327

2.14 AmpC β-lactamase genes detection

Multiplex PCR was performed for the detection of AmpC β-lactamase genes (Perez and Hanson, 2002), using specific primers for PCR amplifications. These primers are listed in Table 2.4. The PCR program combine of an initial denaturation at 94°C for 3 minutes, 25 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 72°C for 1 minute. The last cycle was followed by a final extension at 72°C for 7

minutes. The PCR products were analyzed in 1.5% agarose gel, using the following electrophoresis conditions: 30 minutes, 100V, TAE buffer 1X. GelRed staining was used for visualization amplified products under an UV light. Negative controls were performed with water in place of template DNA.

Table 2.4: Primers used for amplification of AmpC genes. Adapted from Perez & Hanson (2002).

Target (s)	**Primer**	**Sequence (5' to 3')**	**amplicon size (bp)**
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	ACC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
	ACCMR	TTC GCC GCA ATC ATC CCT AGC	
MIR-1, and ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
	EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

2.15. Phenotypic and molecular identification of CTX-M group1 genes

For the sequencing of the CTX-M-1 group, a different set of primers was used. These were F 5'-CTTCCAGAATAAGGAATC-3' and R 5'-CCGTTTCCGCTATTACAA-3' with an annealing temperature of 52°C (Dutour *et al*, 2002). All isolates with a product size of 903 bp were sequenced. The sequencing was done on PCR products as described above.

2.16. Phylogenetic determination of CTX-M-15 β-lactamase *Escherichia coli* producing isolates

Five isolates of *Escherichia coli* were selected to determine their phylogenetic group. PCR amplification was used to determine the presence of two genes (*chuA1*, *yjaA1*) and a fragment of DNA known as TspE4C21. This method is to determine phylogeny as was proposed by Clermont *et al* (2000). The primers sequences and products size are listed in Table 2.5.

Table 2.5: The phylogenetic genes used in this study with primers sequences and amplicon size.

Name	Primer sequences (5'-3')	Amplicon size (bp)
chuA	ChuA.1 GACGAACCAACGGTCAGGAT ChuA.2 TGCCGCCAGTACCAAAGACA	279
yjaA	YjaA.1 TGAAGTGTCAGGAGACGCTG YjaA.2 ATGGAGAATGCGTTCCTCAAC	211
TspE4C2	TspE4C2.1 GAGTAATGTCGGGGCATTCA TspE4C2.2 CGCGCCAACAAAGTATTACG-	152

2.17. Plasmids identification by PCR-based replicon typing

Plasmid replicons were examined using the PCR-based replicon typing scheme proposed previously (Carattoli *et al*, 2005; Garcia-Fernandez *et al*, 2009). Plasmid replicons were examined for the ESBL-producing isolates and for transconjugants obtained after conjugation of some parental isolates. Replicons, primers, and amplicon sizes are listed in Table 2.6.

Table 2.6: Primers and amplicon sizes, and references used in PCR-based replicon typing.

Replicon name	**Primer (5′-3′)**	**Amplicon size (bp)**	**References**
IncR	F) TCG CTT CAT TCC TGC TTC AGC R) GTG TGC TGT GGT TAT GCC TCA	251	Garcia-Fernandez *et al*, 2009
IncI1	F) CGAAAGCCGGACGGCAGAA R) TCGTCGTTCCGCCAAGTTCGT	139	Carattoli *et al.*, 2005
IncFIA	F) CCATGCTGGTTCTAGAGAAGGTG R) GTATATCCTTACTGGCTTCCGCAG	462	Carattoli *et al.*, 2005
IncFIB	F) GGAGTTCTGACACACGATTTTCTG R) CTCCCGTCGCTTCCAGGGCATT	702	Carattoli *et al.*, 2005
IncFII	F) CTGTCGTAAGCTGATGGC R) CTCTGCCACAAACTTCAGC	270	Carattoli *et al.*, 2005
IncN	F) GTCTAACGAGCTTACCGAAG R) GTTTCAACTCTGCCAAGTTC	559	Carattoli *et al.*, 2005
IncL/M	F) GGATGAAAACTATCAGCATCTGAAG R) CTGCAGGGGCGATTCTTTAGG	785	Carattoli *et al.*, 2005

2.18. Pulsed-field gel electrophoresis

PFGE was performed to determine the clonality of bacterial isolates. All of the isolates producing *bla*$_{\text{CTX-M}}$ genes in this study were genotyped using the PFGE protocol suggested by Ejrnaes *et al*, 2006 with some modification (see below). DNA fingerprinting profiles for the isolates were examined digitally with BioNumerics software (Applied Maths, Gent, Belgium).

The isolates were grown on IST agar at 37°Cfor 24h. About 3ml of suspension buffer (10 mM EDTA, 1mM Tris HCL, ph8) were mixed with full loops of growth isolate colonies to obtain an optical density of 1.2 to 1.7 at wavelength of 600 nm. Then the suspension was incubated at 55°C for 10m with 500µl of TE buffer (10 mM Tris HCL,ph8, 100 mM EDTA). The solid agarose plugs were prepared by mixing 500µl of bacterial suspension with an equal volume of 1% low melting Agarose, 1%SDS, and 25µl of proteinase K. The plugs kept in 4°C for 10 min. The plugs were kept in 5ml lysis buffer (50mM Tris HCL,PH 8, 50 mM EDTA, 1% laurylsarcosine) for 2h at 55°C. The DNA plugs were washed twice in distilled water and three times in TE buffer. For each wash, there was a 15 min. incubation period at 55°C. The restriction enzyme Xba1 was then added to the plugs and incubated overnight at 37C. The DNA plugs were put in the well of an agarose gel and then 0.5X TBE buffer was added to cover the wells. The electrophoretic conditions were adjusted as follows; initial pulse time of 0.1s, second pulse time 38s, run time 22h, gradient v/cm. The gel was stained in GelRed and then photographed. The results were analysed using BioNumerics software. Strains

were defined as having a clonal relationship if they possessed similarity of 85% to PFGE profiles using 30U of Xba1 restriction enzyme on a CHEF DRII apparatus.

2.19. Genome Walking and Simplex PCR for genetic environment studies

PCR was used to amplify and sequence the genetic environment surrounding the *bla*$_{\text{CTX-M-15}}$ gene. Specific primers were used for the insertion sequences IS*26* and IS*Ecp1* (upstream) and for the open reading frame orf477 (downstream) in combination with specific primers of *bla* CTX-M-15 (Dhanji *et al*, 2011; Poirel *et al* , 2003; Cullik *et al*, 2010, Pilhofer *et al.*, 2007). The primers sequences and amplicon sizes used in gene walking-PCR in this study are listed in Table 2.7.

Table 2.7: Primers sequences for GW-PCR for the detection of the upstream and downstream environments of *bla*$_{CTX-M-15}$.

	Primer	Sequences (5′-3′)	Isolate No.	Amplicon size (bp)	References
Upstream	F) IS26b R) CTX-M-1rev	ATGAAC CATTCAAAGGCCG AGCTTATTCATCGCC ACGTT	9,10	2400	Dhanji *et al*, 2011
	F) ISEcp1 R) IS26a	CCTAGATTCTACGTCAGTACTTCAA AAA TTACATTTCAAAACTCTGCTTACC	12, 23	1700	Dhanji *et al*, 2011
	F) ISEcp1A R) ISEcp1B	GCA GGT CTT TTT CGT CTC C TTTCCGCAGCACCGTTTGC	25	2500	Poirel, Decousser & Nordmann, 2003
	TSP2_X1_+203 TSP3_X1_+83	CGCTCATCAGCACGATAAAG (only one primer for PCR GCATACAGCGGCACACTTC (for direct sequencing)	60		(Cullik *et al*, 2010:Pilhofer *et al.*, 2007)
Downstream	F) CTX-M-1 for R) orf477	AAA AAT CAC TGC GCC AGT CTG GGA CCT ACG TGC GCC CG	9, 10, 12, 23, 25, 60	1300	Dhanji *et al*, 2011

2.20. Plasmid sizing and grouping

Plasmids sizing was achieved with the prepared plugs of PFGE containing genomic DNA. This method used nuclease S1 (Promega), which converts the circular plasmid into linear structure; and permits accurate sizing by measuring the distance of the DNA migrated in the running gels without the interference of circular DNA. This protocol was suggested previously by Barton (1995). Ten units of nuclease S1 were added to each PFGE plug, followed by 90 μL of sterile distilled water with 10μL nuclease S1 buffer (Promega). The PFGE plugs were incubated for 45 minutes at 37^0C. The plugs washed with sterile distilled water to stop the enzymatic reaction.

For isolates harbouring more than one plasmid, DNA gel extraction for each band was done using QIAquick gel extraction kit (Qiagen). PCR amplification was done for each plasmid, using primers of CTX-M primers, F 5'-CTTCCAGAATAAGGAATC-3' and R 5'-CCGTTTCCGCTATTACAA-3' at annealing temperature of 52°C (Dutour *et al*, 2002) as mentioned above in section 2.15. Further sequencing was made to confirm the existence of *bla*$_{\text{CTX-M}}$ genes as described in part 2.4. PCR-based plasmid replicon typing was done as described (Carattoli *et al.*, 2005, Garcia-Fernandez *et al.*, 2009). The PCR reaction mix gel visualization protocols were made as described above.

2.21. Conjugation studies

The transferability of the plasmids harbouring *bla*$_{\text{CTX-M}}$ genes was done by broth mating. The clinical isolates were used as donors and *Escherichia coli* J62.2 (Rif^{R}) was the recipient. Both had been grown in 4.5ml sterile nutrient broth after overnight incubation at 37^{0}C. One hundred μL of the donor's isolate cultures was added to 1 ml of the recipient isolate (i.e. mixed in the ratio of 1:10 donors:recipient strains) in 4.5 prewarmed 4.5ml sterile nutrient broth and incubated overnight at 37^{0}C. Transconjugants were cultured on MacConkey agar supplemented with rifampicin (50 mg/L) and cefotaxime (32 mg/L) (Amyes and Gould, 1984). Controls were prepared for donor and recipient isolates by inoculation both of them onto MacConkey agar with the same antibiotics. Successful conjugation was confirmed by PCR amplification.

2.22. Integron class 1 gene detection

The primers used for amplification and detection of class 1 integron were previously described by Kerrn *et al* (2002). All *bla*$_{\text{CTX-M}}$-producing isolates were tested for the presence of integron 1 gene (*intI*1) and then sequenced. The primers used for the PCR were: Int-F, 5'GCCACTGCGCCGTTACCACC3'; Int-R, '5GGCCGAGCAGATCCTGCACG 3' (898 bp).

Section C

Chapter - 3: Susceptibility Testing and Identification of Extended Spectrum β-Lactamases (ESBLs)

3.1. Abstract

***Objectives*:** The aim of this chapter was to investigate the prevalence of ESBL producing Enterobacteriaceae species isolated in a hospital in London.

***Results*:** Amongst 100 isolates received from London St George's hospital, there were 50 *Escherichia coli*, 12 *Klebsiella pneuomoniae*, 5 *Klebsiella oxytoca*, 7 *Enterobacter cloacae*, one isolate *Enterobacter aerogens*, one *Enterobacter spp*., 12 *Proteus mirabilis*, one *Proteus vulgaris*, six Lactose Fermenting Coliform, two isolate *Pantoea spp*., one isolate *Morganella morgana*, one isolate *Hafnia alvei*, and one isolate *Serratia marcescens*. The sensitivity of four classes of antibiotics including, third generation cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), carbapenems (imipenem, meropenem, and ertapenem), fluoroquinolones (ciprofloxacin), and aminoglycoside (genetamicin) was examined for MIC. , Six (6%) of these isolates were resistant to cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, and gentamicin. On the other hand, all 100 isolates were sensitive to imipenem, ertapenem, and meropenem. The overall frequency of ESBL producers detected in this study was 6/100 (6%) and most of these (5/6) were from *Escherichia coli* and one isolate was from *Klebsiella pneuomoniae*.

***Conclusion*:** This work reports genotypic analysis and susceptibility profiles of clinical isolates of Enterobacteriaceae from St George's Hospital London.

3.2. Introduction

Enterobacteriaceae have emerged as one of the most significant causes of both nosocomial and community acquired infections. β-lactams (mainly extended-spectrum cephalosporins and carbapenems) and flouroquinolones antibiotics represent the main therapeutic options to treat infections caused by these pathogens (Canton *et al*, 2008).

Extended spectrum β-lactamases (ESBLs) are class A enzymes that have the ability to hydrolyse third generation cephalosporins (ceftriaxone, cefotaxime, and ceftazidime) but are inhibited by clavulanic acid. Most of ESBLs producers are susceptible to cefoxitin and cefotetan but they are not active against cephamycins (Martine´z-Martine´z *et al*, 1996). This study investigated the *in vitro* activity by the MIC of meropenem, ceftriaxone, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, ertapenem susceptibility, and clavulanate synergy with cefotaxime and ceftazidime by disc diffusion.

3.3. Results

A total of 100 Enterobacteriaceae isolates were collected in 2010, from St George's hospital London. From the relevant information provided by the hospital, all isolates were collected from blood samples. The isolates were characterised at source by Vitek 2

Table 3.1: Total number of each Enterobacteriaceae species

Isolate species	Number of isolates
E.coli	50
Klebsiella spp	17
Enterobacter spp	9
Proteus sp	13
Lactose fermenting coliform	6
Pantoea sp	2
Serratia marcescens	1
Morganella morganii	1
Hafnia alvei	1
Total	100

Table 3.2: MIC_{50} and MIC_{90} of *E. coli*, *Klebsiella* spp, *and Proteus* spp

Organism (No. of isolates)	**Antimicrobial agent**	**mg/L**		
		MIC_{50}	MIC_{90}	MIC Range
E. coli 50	Cefotaxime	0.06	0.25	0.03-128
	Ceftriaxone	0.06	0.25	0.015-128
	Ceftazidime	0.12	0.25	0.03-164
	Ciprofloxacin	8	32	0.5-64
	Gentamicin	1	32	0.25-128
	Imipenem	0.03	0.12	0.008-0.5
	Meropenem	0.03	0.5	0.008-0.5
	Ertapenem	0.12	0.5	0.03-0.5
***Klebsiella* spp** 17	Cefotaxime	0.03	0.06	0.03-128
	Ceftriaxone	0.06	0.25	0.015-128
	Ceftazidime	0.12	0.25	0.06-128
	Ciprofloxacin	1	8	0.5-16
	Gentamicin	1	8	0.5-32
	Imipenem	0.015	0.06	0.008-0.25
	Meropenem	0.03	0.06	0.008-0.12
	Ertapenem	0.06	0.5	0.03-0.5
***Proteus* spp** 13	Cefotaxime	0.03	0.12	0.03-0.12
	Ceftriaxone	0.06	0.12	0.015-0.12
	Ceftazidime	0.12	0.5	0.12-0.5
	Ciprofloxacin	2	32	0.5-32
	Gentamicin	0.5	1	0.5-1
	Imipenem	0.015	0.06	0.008-0.12
	Meropenem	0.03	0.06	0.008-0.06
	Ertapenem	0.06	0.5	0.03-0.12

3.3.1 Antimicrobial susceptibility

All of the collected isolates were initially examined to determine the MIC of meropenem to exclude the presence of carbapenemases. None of the 100 isolates had an MIC above the breakpoint thus showing complete susceptibility to meropenem and the presence of carbapenemase activity was not examined further.

The MICs of the antibiotics used and other results for these isolates are shown in the Appendix. The MIC range, MIC_{50} and MIC_{90} values for the individual for all 100 isolates are shown in Table 3.2.

Six isolates (five *Escherichia coli* and one *Klebsiella pneumoniae*) were shown to be resistant to the cephalosporins. The MICs showed that all of these isolates were resistant to ceftriaxone, cefotaxime, ceftazidime, ciprofloxacin, and gentamicin (Table 3.3).

Table 3.3: MICs of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolates (MIC: mg/L), and synergy of both cefotaxime and ceftazidime with clavulanate. Grey colour indicates resistant isolates.

Isolate No.	MIC (mg/L)								Synergy with clavulanate	
	Ciprofloxacin	Ceftriaxone	Cefotaxime	Ceftazidime	Imipenem	Meropenem	Ertapenem	Gentamicin	Cefotaxime	Ceftazidime
9	8	32	64	8	0.015	0.015	0.5	8	+	+
10	8	32	32	8	0.12	0.015	0.5	8	+	+
12	16	>128	64	16	0.008	0.06	0.5	8	+	+
23	16	>128	64	8	0.12	0.12	0.03	16	+	+
25	16	>128	>128	64	0.06	0.008	0.03	8	+	+
60	16	>128	>128	>128	0.06	0.03	0.06	16	+	+

3.3.2. Confirmation of ESBLs production

All isolates that had been found to be resistance to cefotaxime, ceftazidime, and ceftriaxone were subjected to confirmatory tests by the double and combination disc diffusion methods as described previously in the Materials and Methods (2.12) and shown in Figures 3.1. All were found to show positive synergy with cefotaxime plus clavulanate and ceftazidime plus clavulanate, indicating the presence of ESBLs (Table 3.3).

Figure 3.1. Example of synergy by the double disc detection method for ESBL production. Discs: centre, amoxicillin + clavulanate 20 + 10μg; up, cefotaxime 30μg; and down, ceftazidime 30μg

3.3.3. CTX-M genes detection by Multiplex PCR

Multiplex PCR was performed for the identification of *bla*CTX-M genes using specific primers designed for identifying known β-lactamase genes including individual *bla*CTX-M groups 1, 2, 9, and jointly *bla*CTX-M groups 8 and 25. All six isolates had the intrinsic alleles of *bla*CTX-M group 1. The gene encoding CTX-M group 1 β-lactamases is located on an amplicon of 415bp while characteristic amplicons of CTX-M group 2 and group 9 β-lactamase genes were not seen in this study (Figure 3.2 and Figure 3.3).

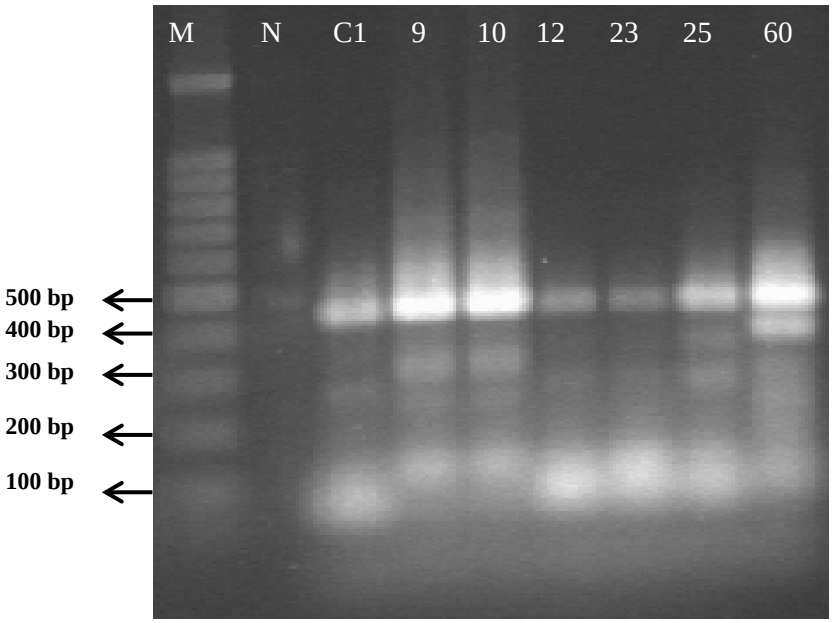


Figure 3.2: Multiplex PCR for CTX-M shows six positive isolates for CTX-M group 1. C1; positive control for CTX-M group1 (415pb); N, negative control; 9, 10, 12, 23, and 25, *E coli*; 60, *K. pneumoniae*; and M, marker (1kb).

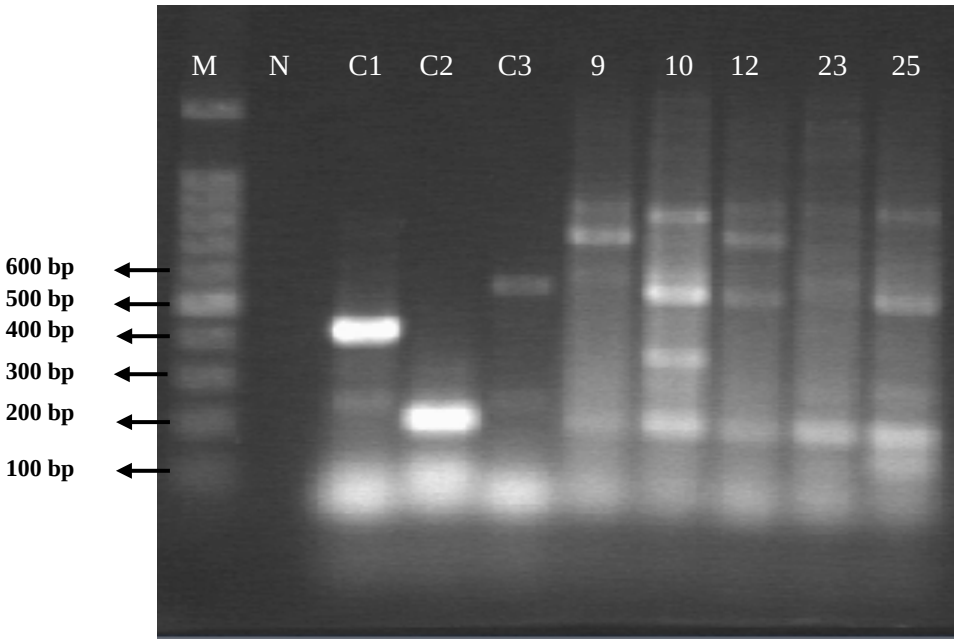


Figure 3.3: Multiplex PCR for CTX-M genes shows examples of five cephalosporin sensitive isolates contain nonspecific sequences along with three CTX-M control genes(C1 = group 1, C2 = group 9 and C3 = group 2), these genes have expected band size of 415 pb for group 1, 552 pb for group 2, and 205 pb for group 9. 9, 10, 12, 23, and 25, *E coli*; 60, *K. pneumoniae*; M=marker (1kb), N=negative control.

3.3.4. *bla*AmpC genes Multiplex PCR

Multiplex PCR was performed to detect AmpC β-lactamase genes (Perez & Hanson, 2002). using specific primers for PCR amplifications. These primers are listed in table 2.4 in the Material and Methods. *bla*AmpC genes were not found in any isolates of this study (Figure 3.4).

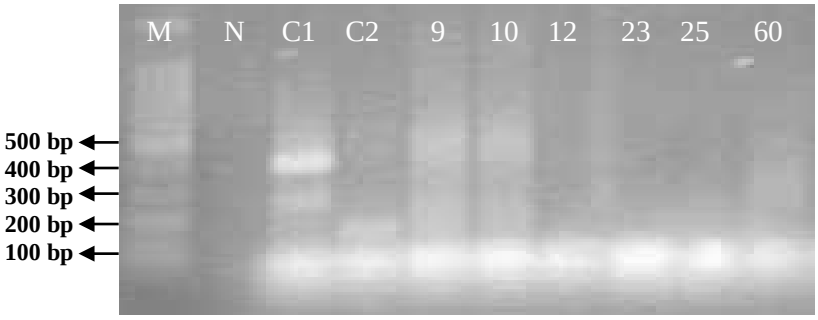


Figure 3.4: Analysis of *bla*$_{AmpC}$ genes in the six cephalosporin resistant clinical isolates. Multiplex PCR products were separated in a 1.5 agarose gel. M, 1 kb DNA ladder; N, negative control (distilled water); C1, positive control with two genes, CIT (462 bp) and EBC (302 bp) family genes; C2, positive control with one gene, FOX (190 pb) family gene; all other isolate shows negative isolates.

3.5. Discussion

The extended-spectrum β-lactamases (ESBLs) are one of the most alarming groups of β-lactamases in clinical practice. There are many reports of increased mortality and deteriorating clinical outcomes for infections caused by bacteria encoding ESBLs (Paterson *et al*, 2004). Bacteria harbouring ESBLs have been isolated from infections, such as septicemia, urinary tract infection, hospital acquired pneumonia, brain abscesses, intra-abdominal abscesses (Mathur, 2002). ESBLs can be defined as β-lactamases capable of donating bacterial resistance to penicillins, first-, second-, and third-generation cephalosporins and monobactams (e.g.aztreonam), but not the cephamycins or carbapenems but can be inhibited by β-lactamase inhibitors such as clavulanic acid. According to the latter definition, functional classification places most ESBLs into group 2Be.

The majority of ESBLs contains a serine at the active site, and belongs to class A according to the Ambler classification (Ambler, 1980). Today, there are >180 TEM type, >130 SHV type, >90 CTX-M type, and 11 OXA type β-lactamases (http://www.lahey.org/studies/). ESBLs produced by Enterobacteriaceae, particularly those of the CTX-M type, are a major problem globally (Paterson and Bonomo, 2005). CTX-M variants have now subrogated TEM and SHV enzymes as the most common ESBLs produced by gram negative bacteria, particularly *Klebsiella pneumoniae* and *E. coli* (Livermore *et al*, 2007). CTX-M predominantly cleaves cefotaxime more than ceftazidime, although some variability has been reported (Bou, 2005), which probably accounts for its recent success.

Different ESBLs exhibit different levels of resistance to third generation cephalosporins (Gupta, 2007). Many do not increase MICs to levels that can be described as resistance while some reduce MICs below the standard breakpoints (sometimes as low at 0.5mg/L) for resistance (Katsanis *et al*, 1994). Clinical failure can be associated with even weak ESBL activities. It is therefore essential to test ESBL detection to identify the start of the ESBL evolution (Gupta, 2007). It is risky to rely on a single screening antibiotic, such as ceftazidime or cefpodoxime, in a screening test (Sturenburgh & Mack, 2003). There have been many failures in both disc diffusion and MIC tests alone to detect ESBLs (Jacoby and Han, 1996). Also, there are reports showing the failure of automated tests for ESBLs detection (Tenover *et al*, 1999). The utilization of more than one cephalosporing group will increase ESBL detection sensitivity (Federico *et al*, 2007). In addition, the reporting of ESBL- producers should state that these isolates are considered resistant to all penicillins, cephalosporins, and aztreonam (CLSI, 2007). More importantly, molecular detection tests are much more sensitive for specific ESBL detection though they may not measure expression. These methods include: specific DNA probes, PCR with oligonucloetide primers oligotyping, ligase chain reaction and sequencing of nucleotide, and PCR followed by restriction fragment polymorphism analysis (Gupta, 2007). In this study, MIC's for three third generation cephalosporins were used and then synrgy dis confirmatory test was used to confirm the presence of fresistance. In addition, multiplex PCR was used to detect the mechanism of resistance.

A third generation cephalosporins resistant *E. coli* and *Klebsiella pneumoniae* are however, not ESBL producers. Some plasmid-encoding AmpC β-lactamases may be acquired by *Klebsiella* spp (Bush, 2008) and *E. coli* (Paterson, 2001). The ESBL confirmatory tests such cases do not give positive results for AmpC enzymes carrying isolates.

The prevalence of ESBL producing species of the family of Enterobacteriaceae varies in different parts of the world. The prevalence of ESBL-producing bacteria has been reported from North America, South America, Africa, Asia, and Europe (Dhillon and Clark, 2012). Data collected from different parts of the world showed the rate of ESBL production was highest among the *Klebsiella pneumoniae* strains from South America, Asia/Pacific Rim, Europe, and North America (44%, 22.4%, 13.3%, and 7.5%, respectively) (Reinert *et al*, 2007; Falagas & Karageorgopoulos, 209). In China, data in 1998-1999 suggested that ESBL-producing *E. coli* and *K. pneumoniae* isolates, ranged from 13% - 15% and >20% respectively. Recent study observed a significant increasing in the ESBLs-producing *Escherichis coli* over the non- ESBLs-producing *Escherichis coli* in rates of 47.9% and 52.1% respectively (Li *et al*, 2012). The situation in Africa is unrelaible due to a lack of comprehensive data, though there is sufficient evidence suggested the prevalence of ESBLs- producing Enterobacteriaceae in this part of the world (Dhillon & Clark, 2012).

In Europe, although there is prevalence of ESBLs in different European countries, the ESBL-producing Enterobacteriaceae is alarming. several European countries have experienced outbreaks associated with ESBL-producing bacteria (Paterson and Bonomo, 2005). There has been a slight decrease in the number of ESBL- producing *K. pneumoniae* in Western Europe, probably because of improvements in infection control and antimicrobial management (Albertini *et al*, 2002). Data reported in 2006 from 31 European countries showed a continuous rise in resistance to third generation cephalosporins since 2000 both in *E.coli* and *Klebsiella pneumoniae* isolates (EARSS, 2006). The latter data showed significant geographical differences from 1% in Estonia to 41% in Romania for *E. coli* and 0% in Iceland to 91% in Romania for *Klebsiella pneumoniae*. A study from Denmark showed the prevalence of ESBL producers was less that 1% (Kjerulf *et al*, 2008). In France, the frequency of different ESBL producing isolates in 2005 was lower that described in previous years such as *Klebsiella pneumoniae* (9.4% versus 3.71%), but had rised for *E. coli* (0.2% versus 2%) (Galas *et al*, 2008).

These data compare with those from isolates in Asia/Pacific Rim, Europe, and Latin and North America, which showed that the proportion of ESBL production in *E. coli* and *K. pneumoniae* was 7.6% and 7.5% respectively during a similar period. Other study from 22 European countries showed the proportion of ESBL production among 794 *Escherichia coli* isolates and 515 *Klebsiella pneumoniae* isolates was 9.8% and 15.5%, respectively (Hack *et al*, 2008). Data collected from tertiary hospitals, similar to St George's, and intensive care units in the United Kingdom reported that the rate of Enterobacteriaceae producing ESBLs increased from 4.8% in 1997 to 7.4% in

2002 (Masterton & Turner, 2006). Additionally, data received from BSAC reported an elevation in bacteraemia cases due to CTX-M producing *Escherichia coli* from 0.9% in 2002 to 8.3% in 2007 and for *K. pneumoniae* from 0.9% to 11.8% over the same time period (www.bsacsurv.org/mrsweb/bacteraemia). This is reflected in my results, where 10% of the *E. coli* strains were cephalosporin resistant and ESBL positive. Although 6% of the *Klebsiella* isolates were cephalosporin resistant and ESBL positive, the percentage cannot be accurate because of the number of strains.

My results also show the association of other resistance determinants with cephalosporin resistance. The following chapters will examine whether these resistance genes are linked to the β-lactamase gene. Multi-resistance provides the bacteria with an obvious selective advantage, but it is also advantageous to the survival of β-lactamase gene as its will be maintained by the use of antibiotics other than the cephalosporins. A report has shown that the use of various antibiotics has been associated with the emergence and persistence of the ESBL-producing Enterobacteriaceae. It demonstrated that the use of quinolones was a risk factor in the emergence of ESBLs dissemination (Kaier *et al*, 2009).

3.6. Conclusion

These findings report the distribution of ESBLs among the clinical isolates obtained from St George's Hospital London. The cephalosporin resistance and extended-

spectrum β-lactamases detected in this work represented a lower rate of resistance than other recent findings worldwide. On the other hand, this study confirms the predominance of CTX-M type ESBLs that are now present elsewhere in the United Kingdom.

Chapter - 4: Prevalence of transferable *bla*$_{\text{CTX-M-15}}$ from hospital-acquired *Escherichia coli* and *Klebsiella pneumoniae* isolates in London

4.1. Abstract

***Objectives*:** This chapter investigated the prevalence and molecular characteristics of transferable *bla*$_{\text{CTX-M-15}}$ from hospital-acquired *Escherichia coli* and *Klebsiella pneumoniae* isolates in London.

Methods: A total of 100 clinical isolates of various isolates of Enterobacteriaceae received from London St George's hospital in 2010. The isolates showed six of them harbouring CTX-M type ESBLs producers. Conujugation was done to determine where the *bla*$_{\text{CTX-M-15}}$ gene was transferable. The isolates and transconjugants were examined for antimicrobial susceptibility by MICs and agar double dilution method. PCR and sequencing were used to detect *bla*$_{\text{CTX-M-15}}$ gene. Phylogenetic analysis was done by multiplex PCR for the five isolates of *Escherichia coli*.

Results: Six (6%) isolates had been found to harbour the *bla*$_{\text{CTX-M-15}}$, five *Escherichia coli* (and three transconjugants) and one *Klebsiella pneumoniae* isolate. In addition, sequencing confirmed the existence of *bla*$_{\text{CTX-M-15}}$ and the conjugation showed the ability of *bla*$_{\text{CTX-M-}}$15 to transfer. Phylogenetic analysis showed A and B2 groups of *Escherichia coli* isolates.

Conclusion: This chapter identified the mobile element responsible for the emergence and spread of the CTX-M-15 β-lactamase in this London hospital.

4.2. Introduction

Enterobacteriaceae harbouring extended-spectrum β-lactamases (ESBLs), specifically those of the CTX-M type, are a problem worldwide, causing both sporadic infections and outbreaks (Paterson & Bonomo, 2005). Some recent studies suggested that the pandemic dissemination of the CTX-M-15 β-lactamase is due to the highly virulent *Escherichia coli* O25:H4-ST131 (Leflon-Guibout *et al*, 2008; Woodford, 2008).

The CTX-M-15 β-lactamase was described in the United Kingdom in 2001 (Mushtaq *et al*, 2003), and was first reported in 1999 in India (Karim *et al*, 2001). The bacterial strains producing enzyme spread rapidly in the United Kingdom since its first emergence in 2001. During that dissemination, the United Kingdom isolates were often associated with different *E. coli* clones that had previously been detected worldwide (Laupland *et al*, 2008).

The *bla*$_{\text{CTX-M-15}}$ gene is also associated with other resistance genes and is mostly located on plasmids, and normally belong to the IncF group (Bonnet, 2004). These plasmids contain more than one replicon and in multireplicon plasmids, one replicon is necessarily conserved, owing of the selective pressure causing duplication of the plasmid, while the other replicon is free to diverge (Sykora, 1992). The IncF plasmids are not, therefore, an homogeneous group of plasmids as they vary in size between 50 to 200kb and have the ability to carry the repFII replicon alone or in association with repFIA or/and repFIB (Carattoli *et al*, 2008).

Interestingly, in the United Kingdom, the IncF plasmids harbouring the *bla*$_{\text{CTX-M-15}}$ gene are not associated with specific clones, such as *Escherichia coli* ST131, but rather they have other *Escherichia coli* sequence type, including ST405, ST354, ST28, and ST695 as well as in other species of the Enterobacteriaceae (Coque *et al*, 2008; Diestra *et al*, 2009; Hrabak *et al*, 2008). Variants of the CTX-M β-lactamase are often confined within localised geographical areas, such as the CTX-M-3 β-lactamase in Eastern European countries, and the CTX-M-9 and CTX-M-14 β-lactamase in both Spain and Portugal (Coque *et al*, 2008; Navarro *et al*; 2007, Novais *et al*, 2007). It should be noted that the latter two are closely related to one another. It is has not been identified why this localised distribution occurs but it is likely to be the result of regional clinical prescribing practices for antibiotics.

4.3. RESULTS

4.3.1 Conjugation results

By using conjugation method listed in the Section 2.21 in Materials and Methods section, transconjugant plasmids were successfully transferred from isolates 9, 10, and 25 of *E. coli* strains. Transconjugants could not be obtained fom the other three isolates, despite repeated attempts including increasing the duration of the experiment.

4.3.2. PCR amplification and sequencing of CTX-M-15 genes.

The overall frequency of ESBLs-producing *Escherichia coli* and *Klebsiella* spp detected in this study had been 5/50 (10%) and 1/17 (6%) respectively. The multiplex PCR had shown the existence of *bla*$_{\text{CTX-M-1}}$ group genes (Chapter 3) in these six isolates. These six isolates and the three transconjugants were further tested by PCR with specific primers for the *bla*$_{\text{CTX-M-1}}$ group. These primers, CTX-M-1A and CTX-M-1B, are listed in 2.15 in Material and Methods section (Dutour, Bonnet *et al*, 2002). Each isolate yielded a 903bp product for CTX-M-15 gene (Figure 4.1). The results observed that CTX-M-1 genes were seen in the six isolates of *E. coli* and *Klebsiella pneumoniae* and three transconjugants of from isolates 9, 10 and 25

All isolates showed the presence of the gene. The PCR products were then sent for sequencing. The nucleotide sequence data was analysed and identical for each of the six isolates and identical to previous submissions to GENBANK for *bla*$_{\text{CTX-M-15}}$. The deduced amino acid sequence was identical with the CTX-M-15 β-lactamase in each of the six strains (Figure 4.2). The common sequence from this study was deposited in the GenBank nucleotide database under accession number AB701573.1.

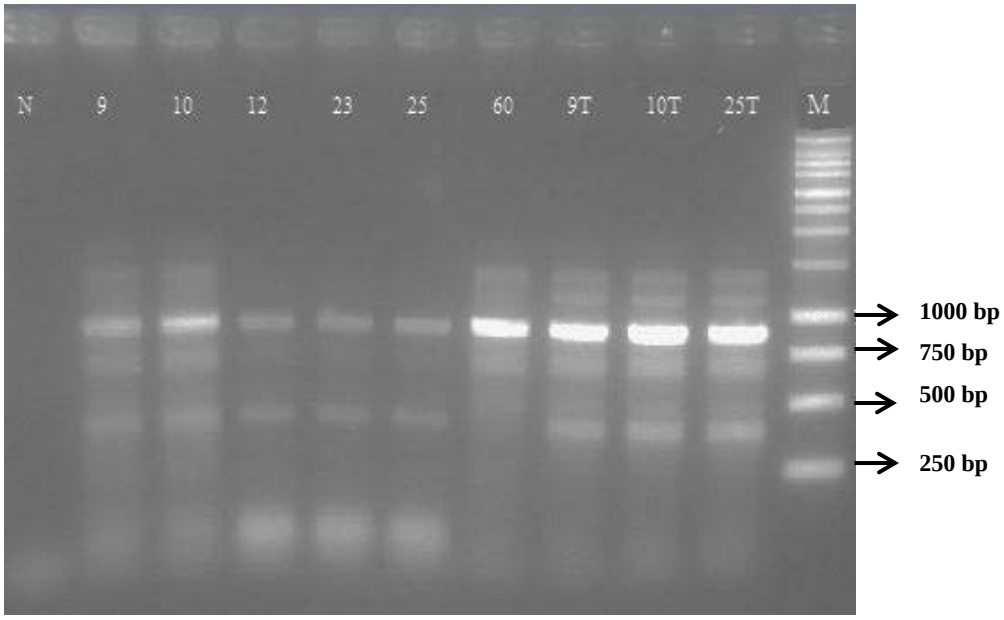


Figure 4.1: PCR amplification of CTX-M-15 β-lactamase genes (903 bp band) for the isolates and transconjugants. The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates. Lane M: 1 Kb marker. Lane N: negative control (free DNA).

Figure 4.2: Nucleotide differences between isolates sharing 98-99% similarity with *bla*CTX-M-15 sequences. Areas in red are identical, black indicates low consensus, and blue indicates neutral consensus.

4.3.3. The β-lactam susceptibility profile

The pattern of resistance to antimicrobial agents in all 6 cefotaxime-resistance *E. coli* and *K. pneumoniae* isolates and their corresponding transconugants (9, 10, and 25) is shown in Table 4.1. The antimicrobial susceptibility patterns revealed that all the isolates that harboured CTX-M-15 ESBLs were characterized by high resistance to all cephalosporins examined. The resistance was transferred with the β-lactamase

gene. Although the co-transfer of gentamicin resistance seen might be expected, the co-transfer of ciprofloxacin resistance was not, indicating the presence of plasmid-mediated quinolone resistance genes. The transconjugants were susceptible to carbapenems as expected (Table 4.1).

Table 4.1: MICs antimicrobial susceptibility test of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolates and their corresponding transconjugants (MIC: mg/L), Grey colour indicates to resistant isolates.

Isolate No.	MIC (mg/L)							
	Ciprofloxacin	Ceftriaxone	Cefotaxime	Ceftazidime	Imipenem	Meropenem	Ertapenem	Gentamicin
9	8	32	64	8	0.015	0.015	0.5	8
10	8	32	32	8	0.12	0.015	0.5	8
12	16	>128	64	16	0.008	0.06	0.5	8
23	16	>128	64	8	0.12	0.12	0.03	16
25	16	>128	>128	64	0.06	0.008	0.03	8
60	16	>128	>128	>128	0.06	0.03	0.06	16
T9	8	32	64	8	0.015	0.015	0.5	8
T10	8	32	32	8	0.12	0.015	0.5	8
T25	16	128	128	64	0.06	0.008	0.03	8

4.3.4. Phylogenetic analysis of *Escherichia coli* isolates

Phylogenetic studies have proposed that *Escherichia coli* may be classified into four main groups or clusters; the more virulent cluster B2 and the low extent cluster D, and cluster A or B1 the commensal strains (Clermont, Bonacors & Bingen, 2000). Although, virulence factors are known to be mobile, a connection between virulence and strain phylogeny has been described (Picard *et al*, 1999). The B2 phylogenetic group represents the majority of strains involved in extraintestinal infections and is deeply divergent from other strains. Such virulence determinants within certain phylogenetic group in a species represent a clonal regardless horizontal gene transfer (Desjardins *et al*, 1995) permits the evolution of an early genetic divergence within the *E. coli* species which may led to descriminate phylogenetic group A and B1 commensal strains, on the one hand, and to phylogentic group B2 of pathogenic strains, on the other hand. The former group of strains emerged as extraintestinal virulence by obtainig of numerous pathogenic determimnants (Lecointre *et al*, 1998). However, there is a stable connection between virulence and phylogeny as virulenc determinants organise into the right genetic background, resulting into certain clone which represents the microbial pathogeneicity in pathogeneic bacteria (Neidhardt *et al*, 1996).

The method of phylogenetic classification of extra-intestinal pathogenic *Escherichia coli* depends on the identification and combination of two preserved genes (*chuA* and *yjaA*) and the DNA fragment TSP (Clermont *et al*, 2000). Therefore each strain was examined by a multiplex PCR for the carriage of the two preserved gene and the DNA fragment TSP. The PCR was run as described in the Materials and Methods

and the amplicons were separated on a gel (Figure 4.3). The results of these three amplifications are tabulated in Table 4.2 and this allowed the creation of a dichotomous decision tree (Figure 4.4) for phylogenetic grouping. From this information, two *E. coli* isolates (9 and 10) were identified as phylogenetic group A and the rest of *E. coli* isolates (12, 23 and 25) were showed group B2.

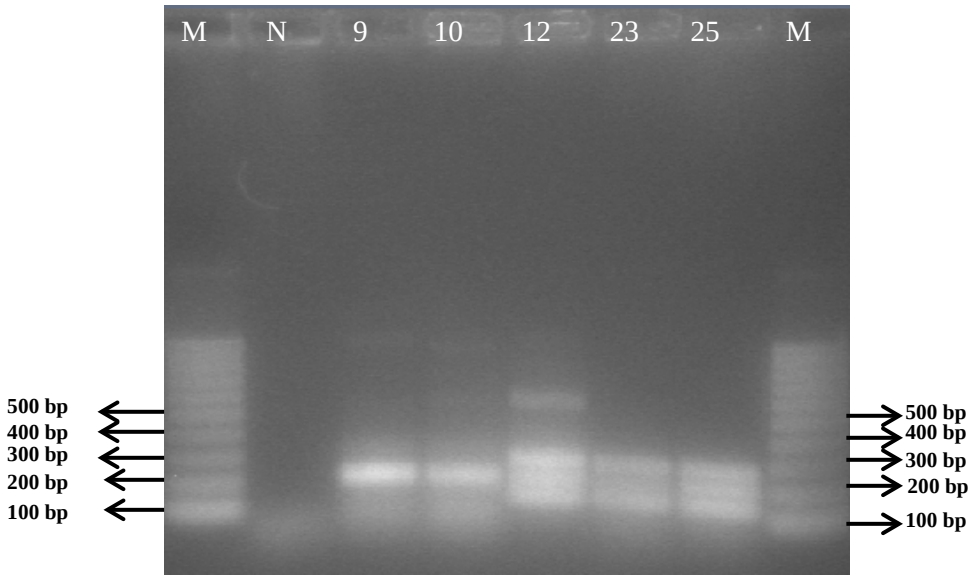


Figure 4.3: Triplex PCR amplification of *chuA* (279bp), *yjaA* (211bp), and fragment TspE4C2 (152bp) for identification of the phylogenetic groups of *Escherichia coli* isolates 9, 10, 12, 23, and 25. Lanes M: 100 Kb marker. Lane N: negative control (free DNA). The isolate numbers are listed at the top of each lane.

Table 4.2: Identification of the preserved genes in *E. coli* to determine phyogenetic class.

E. coli isolate	*chuA*	*yjaA*	TspE4C2
9	-	+	-
10	-	+	-
12	+	+	-
23	+	+	-
25	+	+	-

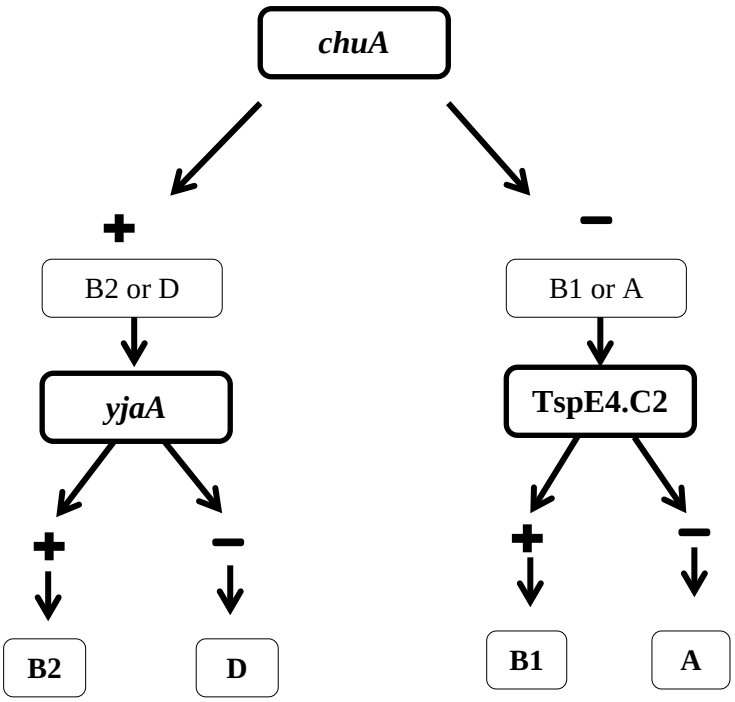


Figure 4.4: Dichotomous decision tree to detect the phylogenetic group of an *E. coli* strain by using the PCR amplification results of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2. (Adapted from Clermont *et al*, 2000)

4.3.5. Discussion

In this study, the CTX-M-15 β-lactamase was the only ESBL found in the six cephalosporin resistant strains in *E. coli* and *Klebsiella pneumoniae*. CTX-M-15 β-lactamase has also been detected in hospitals and community as well as in companion animals in the United States and other parts of the world, (Castanheira *et al*, 2008; Urban *et al*, 2010; Shaheen *et al*, 2011), it is the presence of the CTX-M-15 β-lactamase in European countries that may bear most relevance to this study. A study from Russia identified the *bla*$_{\text{CTX-M}}$ gene in 35.9% of *Escherichia coli* and 34.9% of *Klebsiella pneumoniae* ESBL producing isolates (Edelstein *et al*, 2003). In Austria, among 149 ESBL-producing Enterobacteriaceae isolates obtained between 1998 and 2004, 49 were CTX-M family producers, 38 in *Escherichia coli* and 11 in *Klebsiella* spp. The majority of these CTX-M-producers carried the CTX-M-15 β-lactamase, while the rest harboured CTX-M group 9 β-lactamases (Eisner *et al*, 2006). A study in Poland showed that the prevalence of the CTX-M-15 β-lactamase was high in *Serratia marcescens* and *Escherichia coli* in collection of ESBLs Enterobacteriaceae producers (Baraniak *et al*, 2002). All the studies show that the CTX-M-15 β-lactamase quickly comprised not only a high proportion of the ESBL producing strains but was also highly prevalent in the individual populations as a whole. It is therefore suprising that the proportion of cephalosporin resistance and the concomitant number of CTX-M-15 β-lactamase producers was much lower in St George's Hospital, not least for the fact that the isolation of these bacteria was many years after the published studies above.

The pattern of antimicrobial resistance in in the CTX-M-15 6 β-lactamase containing isolates showed there was cross-resistance to ceftriaxone (6), cefotaxime (6), ceftazidime (6), ciprofloxacin (6), gentamicin (6), meropenem (0), imipenem (0) and ertapenem (0), the proportion that was cross-resistace is shown in parentheses. This high proportion of resistance to majority of the antibiotics is facilitated by the fact that the *bla*$_{\text{CTX-M}}$ genes are frequently present on plasmids harbouring genes that give resistance to multiple antibiotics, including chloramphenicol, aminoglycosides, trimethoprim, sulphonamide, and tetracycline (Bonnet, 2004).

The CTX-M β-lactamases confer high levels of resistance to cefotaxime, ceftriaxone, and aztreonam but often less so to ceftazidime (Bonnet *et al*, 2002; Saladin *et al*, 2002). CTX-M-15 β-lactamase is an exception and is the member of this group that gives the highest resistance to this cephalosporin. This is due to the origin of the CTX-M-15 β-lactamase; it originated from the CTX-M-3 β-lactamase, which does not confer ceftazidime resistance (Poirel *et al*, 2002). The substitution of glycine for asparate at position 240, on the β-3 sheet to the right hand side of the active site improves the catalytic activity against ceftazidime by increasing the rate of binding and hydrolysis of this cephalosporin (DuBois *et al*, 1995). The prevalence of the CTX-M-15 β-lactamase in the UK and in Eastern Europe therefore suggests the use of ceftazidime over other cephalosporins.

ESBL producing isolates are often resistant to different families of antibiotics beside β-lactams including, fluoroquinolones (ciprofloxacin), diaminopyrimidimes plus

sulfonamides (co-trimoxazole) and aminoglycosides (gentamicin, amikacin), which suggest that many other factors could promote the persistence of multi-drug resistant ESBL producer strains and plasmids in both community and clinical settings (Canton *et al*, 2008). High rates of resistance against ciprofloxacin in CTX-M β-lactamase producing clinical isolates have been described in previous studies outside the United Kingdom (Pitout *et al*, 2005; Eisner *et al*, 2006). The *bla*$_{\text{CTX-M-15}}$ gene has been found to be linked to genes conferring resistance to co-trimoxazole, gentamicin, and ciprofloxacin in *E. coli* isolates in Canada (Pitout *et al*, 2007). In my study, the transconjugants were also resistant to ciprofloxacin; a fluoroquinolone resistance gene linked to the *bla*$_{\text{CTX-M-15}}$ gene is unusual in isolates from the United Kingdom.

The majority of the *bla*$_{\text{CTX-M-1}}$ group clusters and the *bla*$_{\text{CTX-M-9}}$ clusters belong to phylogenetic groups B2 and D, respectively (Pitout *et al*, 2005; Karisik *et al*, 2008; Lee *et al*, 2010) so it was unusual to find two strains in group A. In addition, the pathogenic strains causing bacteraemia or urinary tract infection mainly belonged to groups B2 and D (Russo and Johnson, 2000; Duriez *et al*, 2001; Lee *et al*, 2010), which correlates well with this study as the isolates were all obtained from blood samples and the majority of these strains belonged to phylogenetic group B2. Another study suggested that CTX-M-producing *Escherichia coli* were more associated to the rare D2 genotype (Branger *et al*, 2005). However, it was reported that the B2 epidemic strains are the predominant strains disseminating the CTX-M-15 β-lactamase in the United Kingdom (Karisik *et al*, 2008). My isolates were obtained from a population living in an area of London (Tooting) with diverse origins, in particular there is mass movement between Tooting and India and

Pakistan. One study from India suggested that phylogenetic groups D and B2 are predominant (Guiral *et al*, 2011), whereas a later study by the same group from India identified that phylogenetic group B2 was the commonest, while group A was the second (Guiral *et al*, 2013). In Pakistan, it has been found that phylogenetic group A was the most frequent followed by phylogenetic group B2 (Saeed *et al*, 2009). This later study could explain my results for samples 9 and 10 which were identified as phylogenetic group A.

4.5. Conclusion

This study has demonstrated that the gene of *bla*$_{\text{CTX-M-15}}$ can be transferred by conjugation and the plasmid harboured resistance determinants to gentamicin and ciprofloxacin. In addition, phylogenetic analysis showed that my isolates belong to Group A and B2.

Chapter – 5: Variations in genetic environments in conjugative IncFI plasmids carrying CTX-M-15 among *Escherichia coli* and *Klebsiella pneumoniae* ESBL producing isolates at St George's Hospital London

5.1. Abstract

***Objectives*:** To identify the molecular epidemiology of CTX-M-15 type ESBL producing *Escherichia coli* (5) and *Klebsiella pneumoniae* (1) isolates at St George's hospital London.

***Results*:** Examination of the six harbouring *bla*$_{\text{CTX-M-15}}$ alleles revealed two phylogenetic groups A and B2. The analysis of PFGE-based patterns showed that only two *Escherichia coli* isolates have more than 85% clonal similarity, while the rest were less similar. The transferable cefotaxime resistance was manifested by plasmids size ranging from 78.0 kb and 152.0 kb. In these isolates, conjugative IncFIA and IncFIB plasmids were detected in 5/6 and 4/6 of the ESBL-type CTX-M producers respectively. Analysis of the genetic environments showed that there were five different genetic arrangements surrounding the *bla*$_{\text{CTX-M-15}}$ gene including various truncations of IS*Ecp1*.

Conclusion: The *bla*$_{\text{CTX-M-15}}$ gene was found in five different genetic arrangements through various length of truncated IS*Ecp1*. The presence of IS*Ecp1* indicates acquisition of the gene perhaps from outside the United Kingdom but the variation suggests in the genetic environment and the strain genotype indicates that direct cross-infection has not been responsible for the spread of the gene.

5.2. Introduction

Bacterial plasmids carry non-essential accessory genetic structures and have varying size from few to hundreds of kilobases. In order to be auto-transferable, a plasmid needs to be greater than about 30kb and they have to be greater than 20kb to carry insertion sequences. Auto-transferable plasmids, passing through different strains, pick up transposons largely through the action of insertion sequences and this has been the main mechanism for the acquisition of antibiotic resistance genes. There is an abundance of insertion sequences in auto-transferable plasmids. The recognition and localization of both full length insertion sequences and fragmented sections identifies how the modular assemblies of genes are formed (Hu & Derbyshire, 1998).

Genes harbouring ESBLs are linked to certaingenetic elements. Specific mobile genetic structures, insertion sequences, transposons, and integrons, have had important roles in the spread of ESBL genes (Poirel, Naas & Nordmann, 2008). However, compared with the spread of TEM and SHV ESBL genes, the dissemination of CTX-M-type ESBL genes are linked to more complicated mobile structures (Canton & Coque, 2006). This may be because the TEM and SHV ESBL genes were mainly derived from *Escherichia coli* (Livermore, 1995) and *Klebsiella pneuomoniae* (Babini & Livermore, 2000) respectively and therefore did not have species barriers to overcome; while the CTX-M genes had to migrate from *Kluyvera* species and this will inevitably required more sophistaicated genetic manipulations in order that the genes could migrate to *Escherichia coli* and *Klebsiella pneuomoniae*.

5.3. Genotyping by Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) (Schwartz *et al*, 1982) separates chromosomal DNA fragments larger than 50kb, created by endonuclease digestion, with two alternating electronic fields. It is thus a useful technique to determine the degree of relatedness between different strains of the same species (Correia, Martin, and Castro, 1994) and is considered to be the "gold standard" for strain typing and bacterial phylogeny. It can also provide information on the degree and frequency of horizontal gene transmission and gene conversion, molecular organization of chromosome, and genomic re-arrangements (Lahti, 1996).

In this study, PFGE typing was used to analyse the six cephalosporin-resistant isolates (5 *E. coli* and one *Klebsiella pneumoniae*) obtained from St George's hospital London. Figure 5.1 shows the PFGE profile of the six strains and it revealed that only two strains were similar (96%) while the others showed less than 85% similarity to these two isolates and to each other. The dendrogram analysis shows six clusters in Figure 5.2.

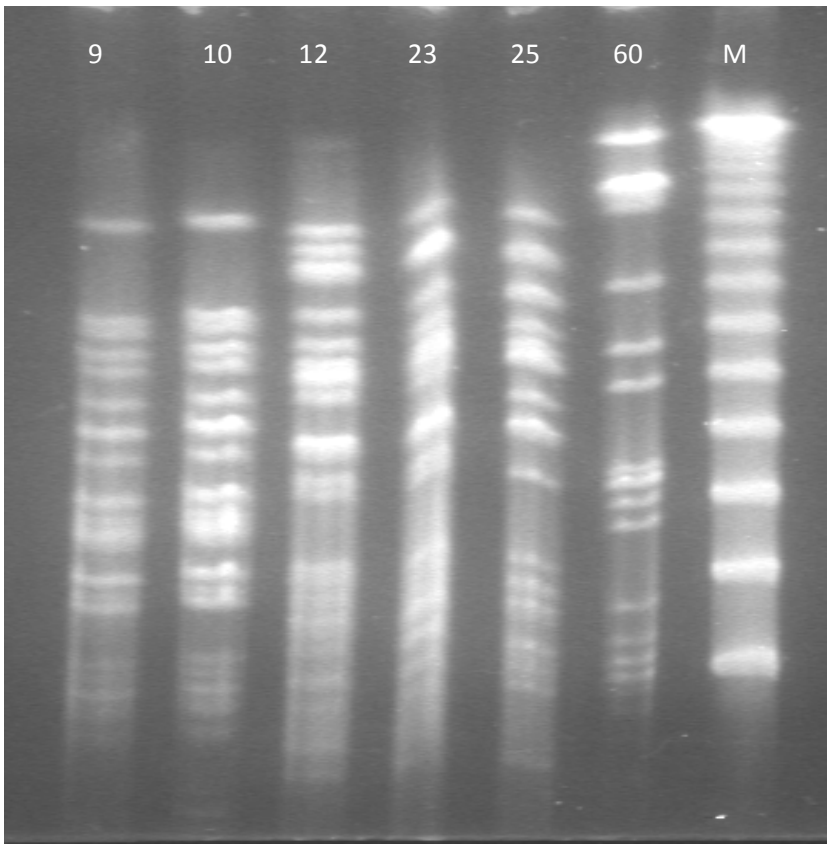


Figure 5.1: PFGE patterns of 5 *E. coli* and one *Klebsiella pneumoniae* clusters 9, 10, 12, 23, 25, and 60. Lanes M: Lambda ladder PFG markers standard size. The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60,

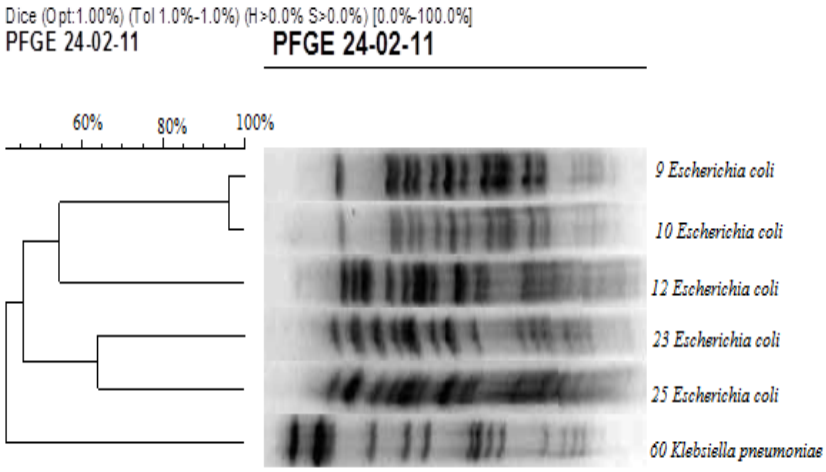


Figure 5.2: Dendrogram analysis of six isolates. Dendrogram showing the relationship between isolates of five strains of *Escherichia coli* and one strain of *Klebsiella pneumoniae* obtained after *Xba1* digestion of the chromosomal DNA.

5.4. Plasmid identification by PCR-based replicon typing for CTX-M-15 plasmids

The ability to describe plasmids accurately is an essential part of epidemiologic studyand healthcare management, particularly during the epidemic plasmids spread in the population. This helps to take appropriate infection control measures (Coque, Baquero & Canton, 2008). The ability to classify and recognise plasmids in homogenous groups according to phylogenetic similarities is essential to determine their dissemination in nature and their evolutionary origin (Francia, *et al*, 2004) and

also to track the dissemination of plasmids donating antimicrobial resistance (Anderson *et al*, 1977). The scheme of a plasmid classification based on the plasmids stability during conjugation, process named plasmid incompatibility (Inc) (Datta and Hedges, 1971). Plasmids with related replication control system are known to be incompatible with each other in the same cell (Datta and Hedges, 1971). Carattoli *et al* (2005) instigated a PCR based replicon typing for the identification of Inc groups and recognised 27 different Inc groups in Enterobacteriaceae (Carattoli 2009) Several studies have reported that many plasmids in the Enterobacteriaceae either have replicons of the IncFII group, or multi-replicons of IncFII linked with IncFIA and/or IncFIB (Gonullu *et al*, 2008).

In this study, PCR amplification was done using primers listed in Table 2.6 in the Materials and Methods to detect replicons of five plasmid incompatibility groups (IncI1, IncFIA, IncFIB, IncFII, and IncR) according to Carattoli *et al* (2005). The results showed the presence of replicon IncFIA in all isolates, except isolate 60, and in each of the transconjugants (Figure 5.3). Replicon IncFIB was found in isolates 9, 10, 12 and 23 and in the transconjugants from isolates 9 and 10. Replicon IncFIB was not found in isolates 25 and 60, nor of course in the transconjugant of isolate 25 (Figure 5.4). Isolate 60 was a *Klebsiella pneumoniae* strain, from which it is often difficult to identify Inc groups by the ordinary PCR based replicon typing. Therefore when S1 nuclease was used to isolate plasmids from this strain, the Inc typing was repeated (see below).

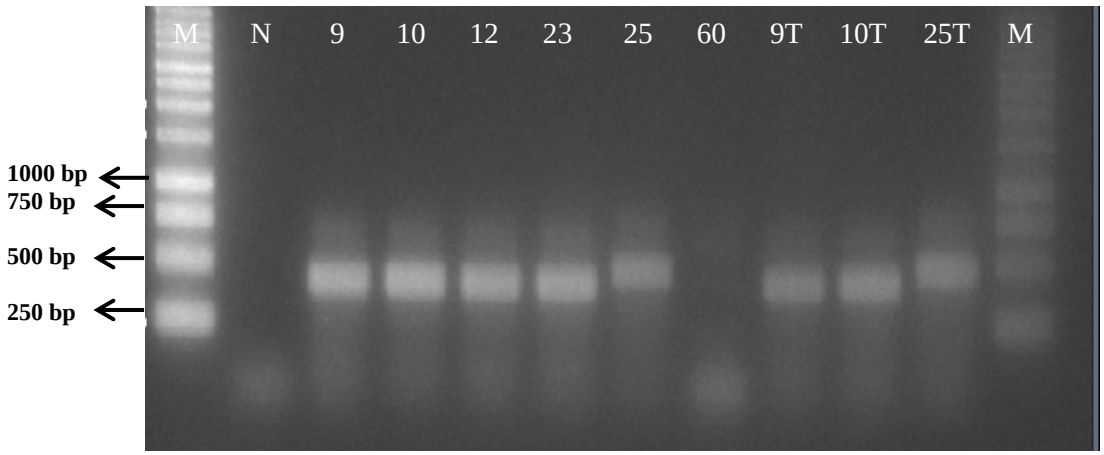


Figure 5.3: PCR amplification of IncFIA replicon (462 pb). Lanes M: 1 Kb marker. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates.

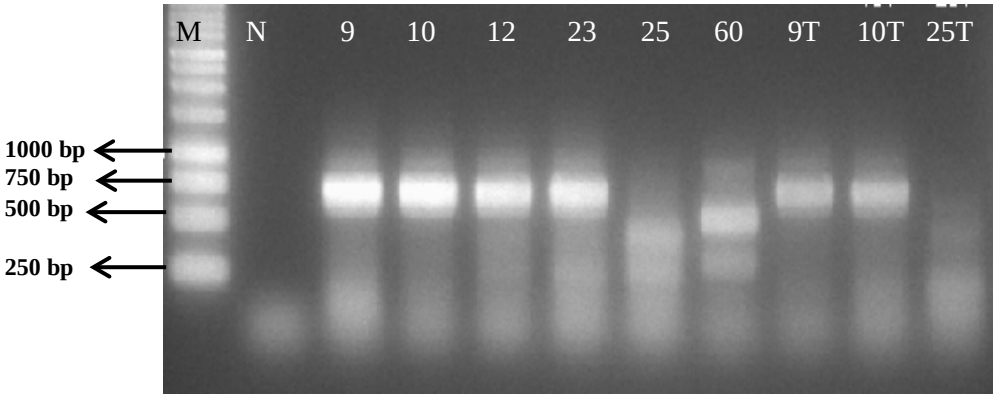


Figure 5.4: PCR amplification of IncFIB replicon (702 pb). Lanes M: 1 Kb-pb markers. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and

K. pneumoniae strain 60, Those that have the suffix T are transconjugants from these isolates.

5.5. Determination of plasmid profiles by Nuclease S1 digestion

Plasmids greater than 100kb in size are called megaplasmids, representing about 10-20% of the genetic material of the bacterium. Large plasmids make a significant contribution to the genomic polymorphism of bacteria, genetic horizontal transfer, and plasmid evolution, so a technique for identifying and sizing the plasmid DNA is often crucial. These megaplasmids are too large to determine in conventional gel electrophoresis, as there are naturally covalently closed-circular supercoiled molecules and their migration is unpredictable in PFGEunlike Linear DNA, which migrates more predictably in a PFGR gel and thus could be used for accurate size determination. When S1 nuclease is added, it nicks the supercoiled plasmids to release linearized DNA (Germond, Vogt & Hirt, 1974). When linear plasmid DNA is separated by PFGE, it can provide an accurate measurement of size when compared against suitable markers (Barton *et al*, 1995).

In this study, each plasmid from both from the isolates and the transconjugants, which had undergone PCR-replicon typing, was extracted from the gel and digested with S1 nuclease and run on a PFGE gel. Furthermore, plasmid number and sizing was also done on the five *Escherichia coli* isolates and one *Klebsiella pneumonia* isolate as well as all associated transconjugants with the total DNA treated with S1 nuclease.

The plasmid sizes are shown in Figure 5.5. All bacteria strains had one detectable plasmid except one *Escherichia coli* strain and *Klebsiella pneumoniae* strain, both of

which possessed three plasmids of different sizes. The co-existence of Inc FIA and FIB replicons were found in all plasmids isolates except one plasmid belonging to *Escherichia coli*, *Klebsiella pneumoniae* and one of the transconjugants. Inc FIA replicon was found alone in one plasmid of *Escherichia coli* a transconjugant. Inc FIB replicon was found alone only in one transconjugant. Two plasmids of *Escherichia coli* and one of *Klebsiella pneumoniae* did not have incompatibility groups identifiable with the primers used.

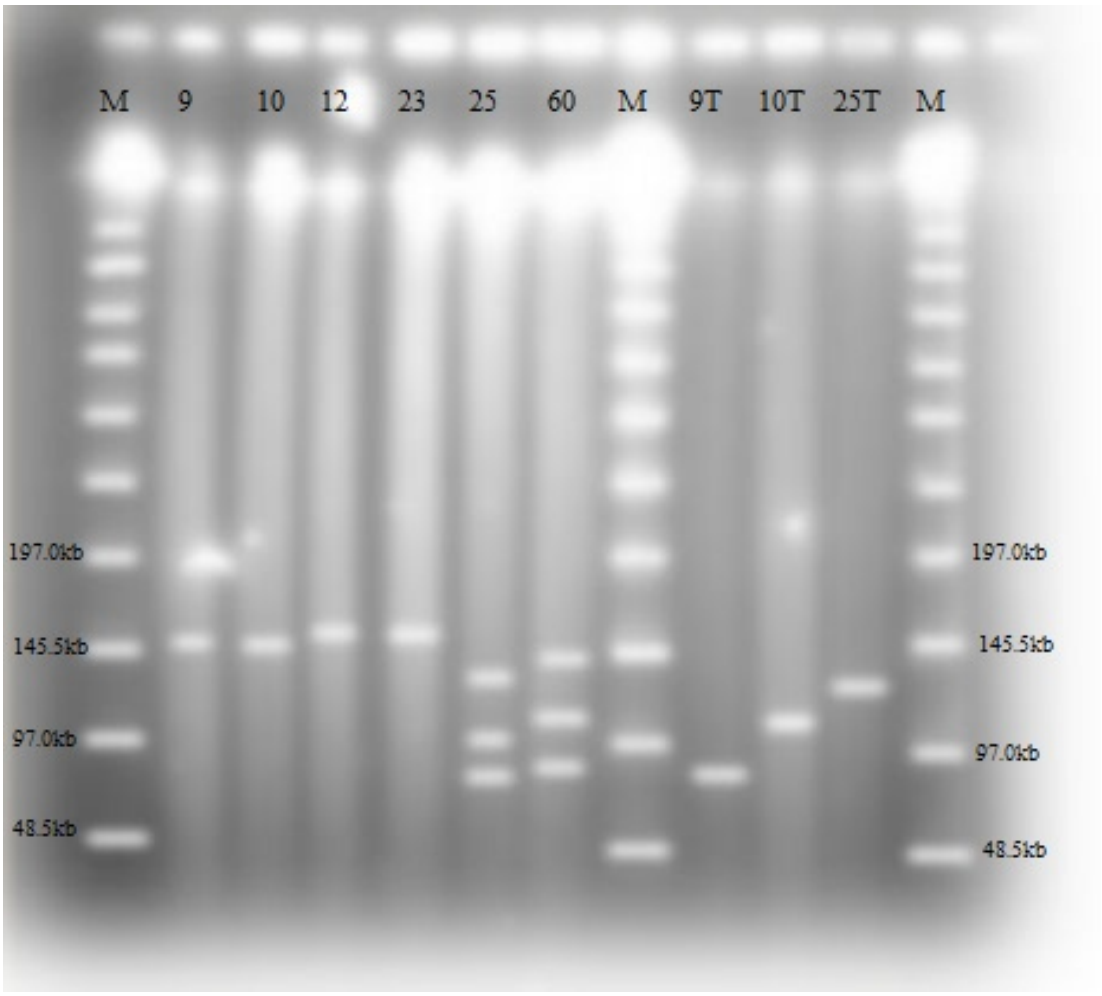


Figure 5.5: Agarose gel showing S1 nuclease PGFE based sizing of large plasmids for 9 isolates. M (Size Marker) refers to the molecular weight marker of the bacteriophage lambda genome. The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates. Plasmids shows variable sizes ranging from 152.0kb to 78.0kb of isolates 9, 10, 12, 23, 25, and 60; and transconjugant isolates T9, T10, and T25.

Table 5.1: Plasmid sizes and incompatibility grouping of the 6 *bla*$_{CTX-M-15}$ containing strains and the transconjugants.

Isolate No	Species	Gene location	Plasmid size (kb)	CTX-M-15 allele	Inc grouping (Inc)
9	*E. coli*	Plasmid	147	+	F1A, FIB
10	*E. coli*	Plasmid	147	+	F1A, FIB
12	*E. coli*	Plasmid	152	+	F1A, FIB
23	*E. coli*	Plasmid	152	+	F1A, FIB
25	*E. coli*	3 Plasmids	129 96 78	+ - -	F1A - -
60	*K. pneumoniae*	3 Plasmids	137 106 81	+ - +	F1A, FIB - F1A, FIB
9T	*E.coli* J62-2	Plasmid	78	+	FIB
10T	*E.coli* J62-2	Plasmid	106	+	F1A, FIB
25T	*E.coli* J62-2	Plasmid	127	+	F1A

The *bla*$_{CTX-M-15}$ allele was observed on a single plasmid found in each strain. However, this gene was found on two plasmids in one strain with multiple plasmids Table 5.1 and Figure 5.6. It is important to note that the plasmid sizes in the three transconjugants did not match the sizes of the plasmids in the original strains. This was a repeatable result on a number of occasions and may reflect either plasmid rearrangement or, more likely, that some of the plasmids in the original isolates were masked during the extraction procedure. What is particularly important to note that a

single plasmid does not seem to be responsible for disseminating the cephalosporin resistance determinant. This is confirmed in the Figure 5.6 which shows that the *bla*CTX-M-15 gene is being carried on plasmids of different sizes.

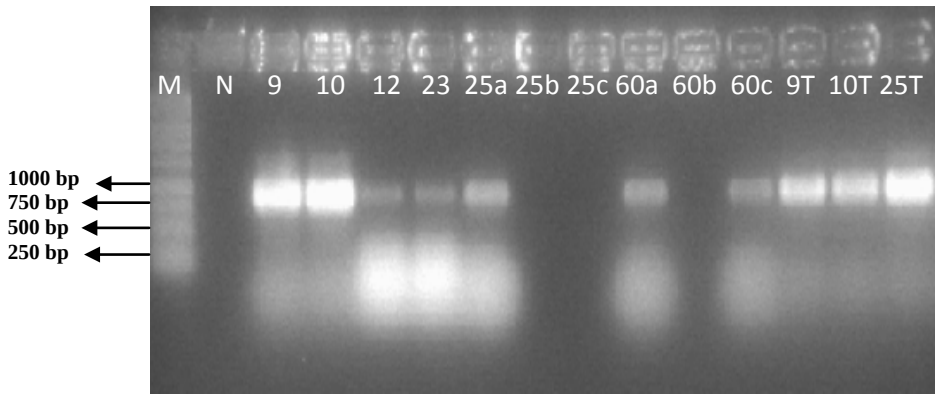


Figure 5.6: PCR amplification of CTX-M-15 β-lactamase genes (903 bp band) of plasmids for the isolates and transconjugants. Lane M: 1 Kb marker. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. Those that have the suffix T are transconjugants from these isolates. Those with a, b, and c indicate multiple plasmids of the isolates ranging from large to small.

5.6. Genome Walking and Simplex PCR for genetic environment studies

Insertion sequences such as *ISEcp*1 have been associated with *bla*$_{\text{CTX-M-15}}$ (Karim *et al*, 2001), often these are located upstream of the *bla*$_{\text{CTX-M-15}}$ genes. Furthermore, the expression of *bla*$_{\text{CTX-M-15}}$ genes needs the presence of a promoter and these insertion sequences upstream of *bla*$_{\text{CTX-M}}$ genes may provide one, though their major role is in the mobility of the gene (Lartigue *et al*, 2006). Studies have shown that there is a variety of flanking regions surrounding these genes (Cantón and Coque, 2006; Carattoli, 2011;Woodford *et al*, 2011); however most comprise just small variations of a coomon genetic backbone (Lartigue *et al*, 2004; Eckert *et al*, 2006; Dhanji *et al*, 2011b; Toleman and Walsh, 2011). These have undoubtedly enabled the spread of the gene as they have contributed to it selective advantage (Baquero, 2004; Cantón and Ruiz-Garbajosa, 2011; Canton, Gonzalez-Alba, and Galan, 2012).

I*SEcp*1 is the most frequently detected insertion sequences upstream of *bla*$_{\text{CTX-M}}$ genes. Although further insertion sequences have been described upstream of the *bla*$_{\text{CTX-M}}$ genes, such as IS*CR*1, IS*10* and IS*26*, Other insertion sequences elements have also been identified upstream but they were the result of integration events. For example, IS10 and IS1, IS*26* and even IS*CR*1, have been shown to insert in and interrupt I*SEcp*1 (Eckert *et al*, 2006; Bae *et al*, 2008). Although it was first found joined to the *bla*$_{\text{CTX-M-15}}$, it has been detected associated with all groups of β-lactamases except CTX-M-8 (Barlow *et al*, 2008).

The spacer sequences between I*SEcp*1 and *bla*CTX-M genes are related to the MICs of cephalosporins (Ma *et al*, 2011). These genetic distances ranged from 48 to 127 bp in *bla*CTX-M-15 gene cluster (Canton, Gonzalez-Alba & Galan, 2012). When the homology among Genetic spacer sequences of *bla*CTX-M-1 genes cluster was examined, all except *bla*CTX-M-10 and *bla*CTX-M-53 maintained a common sequence region. Thus, these *bla*CTX-M genes of *bla*CTX-M-1 group might be originated from a single transposition event (Canton, Gonzalez-Alba, and Galan, 2012).

The genetic environment of the *bla*CTX-M-15 gene in the six isolates was examined the flanking regions were sequenced. This was obtained by initial sequencing of the gene *bla*CTX-M-15 gene and then using this sequence to design new primers, sequencing the upstream region and downstream region as well. The primers used for gene walking detection were listed in Table 2.7 in the Materials and Methods section. These PCR amplifications are shown in Figures 5.7, 5.8, 5.9 and 5.10.

Interestingly, these genetic arrangements show high similarity which indicates the circulation of a certain plasmid harbouring *bla*CTX-M-15 gene in the hospital wards. These findings were confirmed by the existence of the both IncF1A and IncF1B in the six isolates.

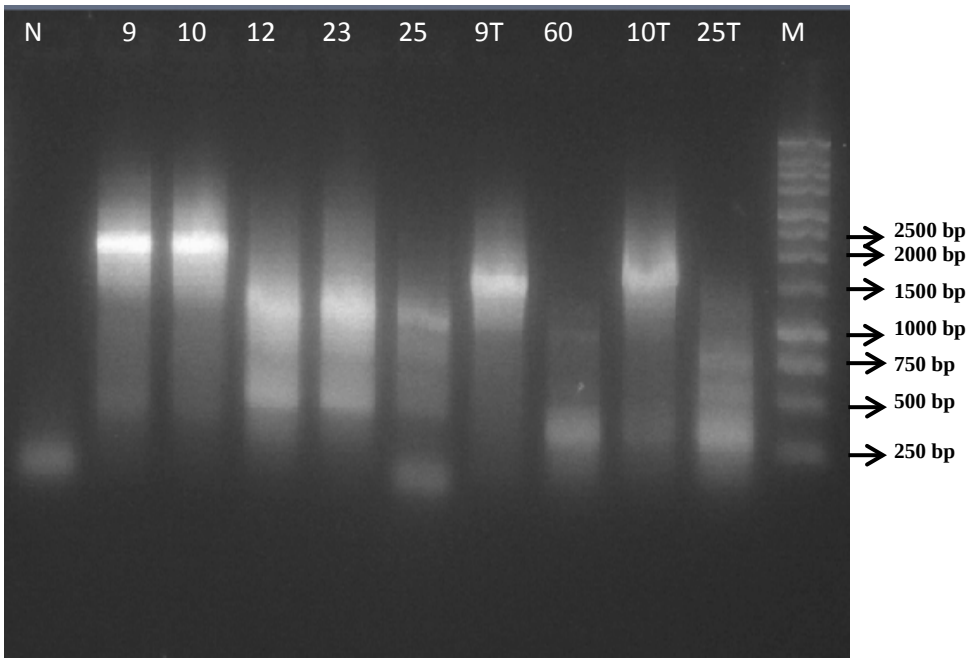


Figure 5.7: PCR amplification of genetic environment positive isolates 9 and 10 (2400 bp), and positive isolates 9T and transconjugant 10T (1600 bp). Lanes M: 1 Kb marker. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates.

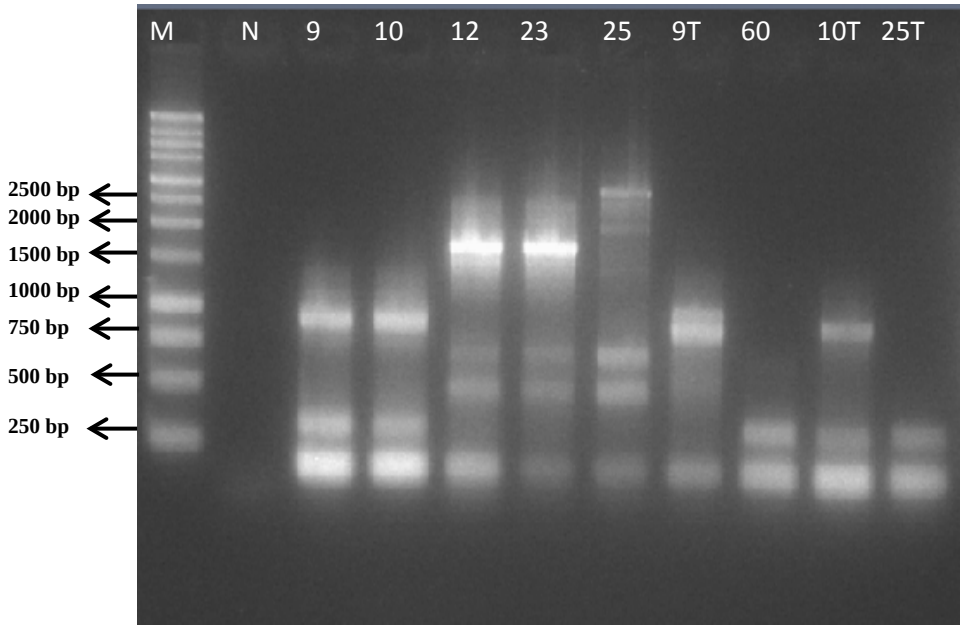


Figure 5.8: PCR amplification of genetic environment positive isolates 12 and 23 (1700 pb). Lanes M: 1 Kb-pb markes. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates.

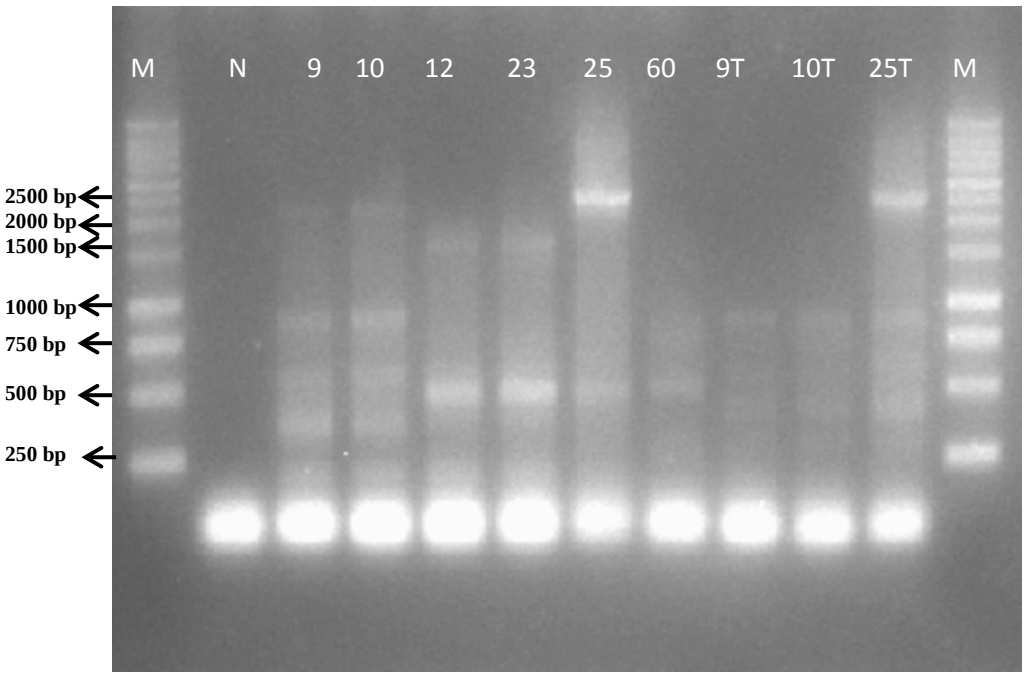


Figure 5.9: PCR amplification of genetic environment positive isolates 25 and 25T (2500 pb). Lanes M: 1 Kb marker. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates.

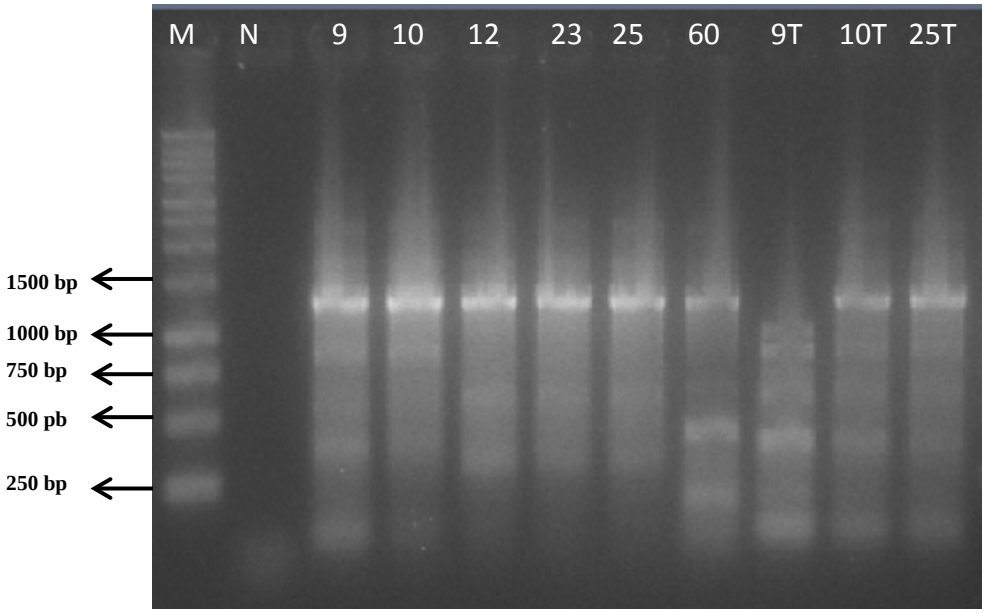


Figure 5.10: PCR amplification of genetic environment (downstream) positive isolates 9, 10, 12, 23, 25, 60, 10T, and 25T (1300 pb). Lanes M: 1 Kb marker. Lane N: negative control (free DNA). The isolate numbers are shown at the top of each lane. The respective isolates are *E. coli* 9, 10, 12, 23, and 25, *E coli* and *K. pneumoniae* strain 60, Those that have the suffix T are transconjugants from these isolates.

The variable region has been sequenced in each isolate and the data are shown in Figure 5.11 and the comparisons are shown diagrammatically in 5.12. It should be noted that orf477 was found downstream in every case; the purpose of this gene is unknown and further downstream studies were not undertaken.

The orf477 was found in different lengths in each isolate; 129 bp in isolates 9 and 10, 135 bp in isolates 12 and 25, and 127 bp in isolates 23 and 60 (Figure 5.12)

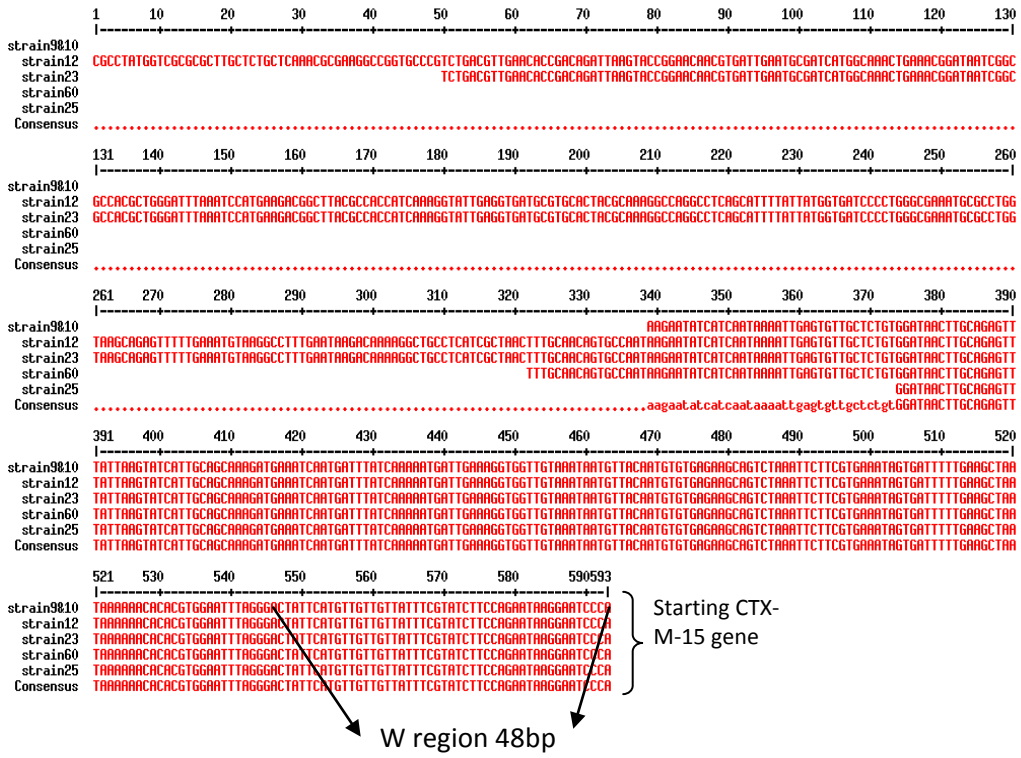
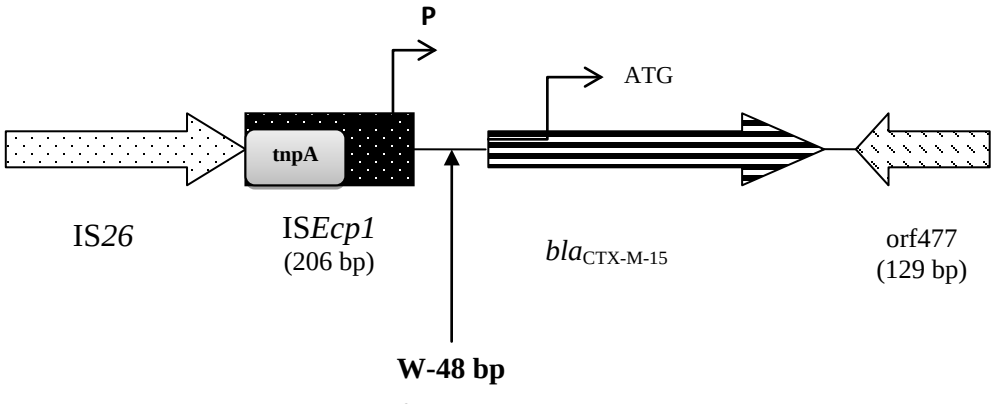
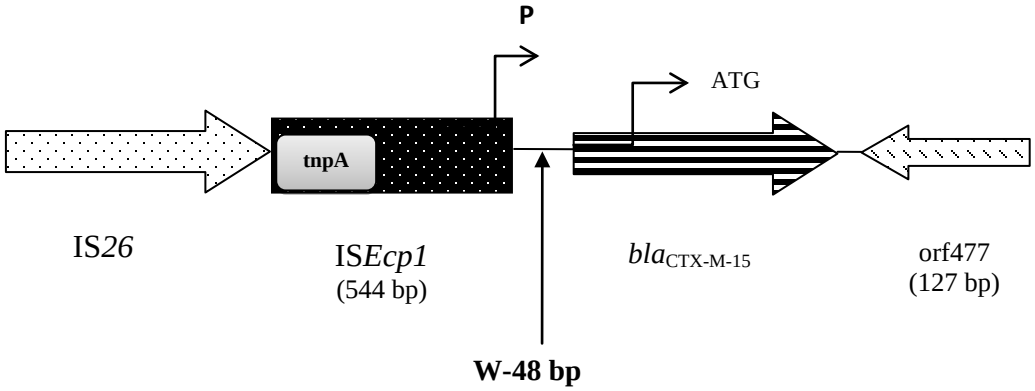


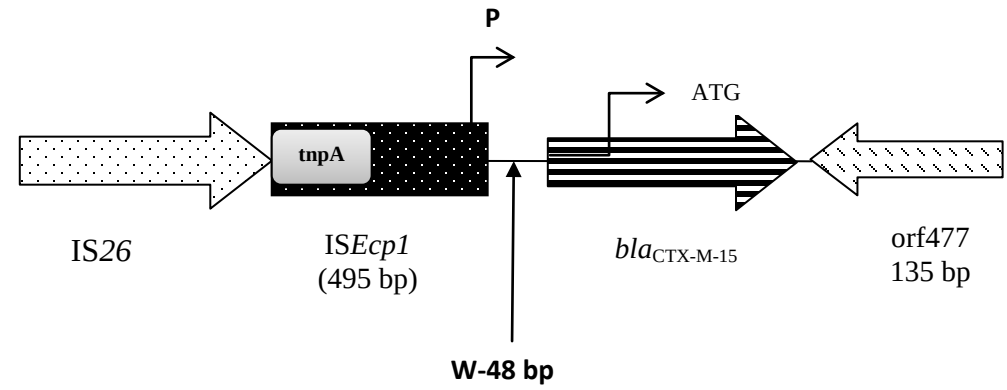
Figure 5.11: Insertion sequences differences size of each isolate of the upstream sequences. Areas in red are identical.



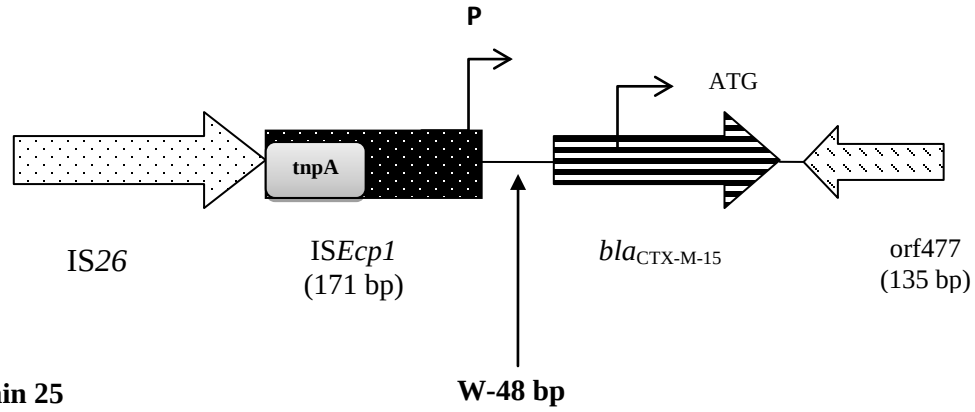
Strain 9, 10



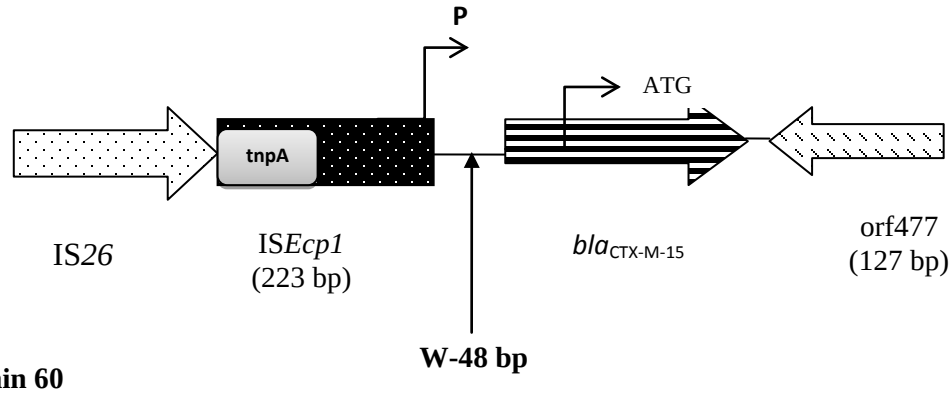
Strain 23



Strain 12



Strain 25



Strain 60

Figure 5.12: Schematic representations of six environments surrounding *bla*$_{\text{CTX-M-15}}$ in five *E .coli* and one *Klebsiella pneumonia* producing ESBL isolates.

A summary of all isolates examined by gene walking primers, GenBank No., IS*Ecp1* size, W region size, and promoters are listed in Table 5.2.

Table 5.2: Upstream structure size for each isolates examined by gene walking primers with "W" region size, and GenBank of upstream and downstream structures.

Isolate No.	GenBank accession No.	IS*Ecp1*			W region size (bp)	orf477
		Size pb	-35 TTGAAA	-10 GCAAAG		
9	HQ157356.1	206	+	+	48	TQ4325591
10	HQ157356.1	206	+	+	48	TQ4325591
12	HQ157353.1	544	+	+	48	TQ4325591
23	HQ157353.1	495	+	+	48	TQ4325591
25	JN788267.1	171	+	+	48	TQ4325591
60	HQ157356.1	223	+	+	48	TQ4325591

The "W" region or spacer (48 bp) was also analysed in this study. The results showed the presence of two promoters in each "W" region of each isolates. These promoters were TTCATG and GCAAAG. The GenBank accession number was AB683465.1.

5.7. Integrons carriage by *Escherichia coli* isolates producing ESBLs enhancing resistance

Integrons are elements which comprise the genetic determinants of a site specific recombination system able to identify and catch mobile gene cassettes. This element contains integrase gene (*int*) adjoining recombination site gene (attI) and a promoter. In addition, there is a part of integron called cassette which does not necessarily exist automonously, but when integrated, become part of the integron (Hall & Collins, 1995). Usually, gene cassette requires a promoter for its expression. So, if the integrated cassette is the close to the promoter, the highest rate of expression will be given in the integron (Collis & Hall, 1995).

Two main groups of integrons have been reported; chromosomal integrons and mobile integrons. Chromosomal integrons are present in most bacterial species chromosome (Cambray, Guerout & Mazel, 2010). Some are found in bacteria such as *Vibrio* spp. and *Xanthomonas* spp. As these bacteria carry more than 200 cassettes, they have been termed superintegrons. On the other hand, mobile integrons are not autotransferable elements but have to be placed within mobile genetic elements such as plasmids and transposons. Moreover, mobile integrons usually compose a limited number of gene cassettes (Naas *et al*, 2001).The gene cassettes revealed in these mobile integrons often encode antibiotics resistance determinants. Therefore, mobile integrons are sometimes termed resistance integrons (Stalder *et al*, 2012). Three classes of resistance integrons (1 – 3) have been described (Hall and Collins, 1995). The majority of resistance integrons belong to class1 which have been observed in

many Gram-negative bacteria including *Escherichia coli* (Schmitz *et al*, 1999) and *Klebsiella pneumoniae* (Girlich, *et al*, 2000).

This study showed one isolate with integron class1 with its transconjugant (Figure 5.13) using primers listed in 2.22 in the Material and Methods section and the amplicons were then sequenced.

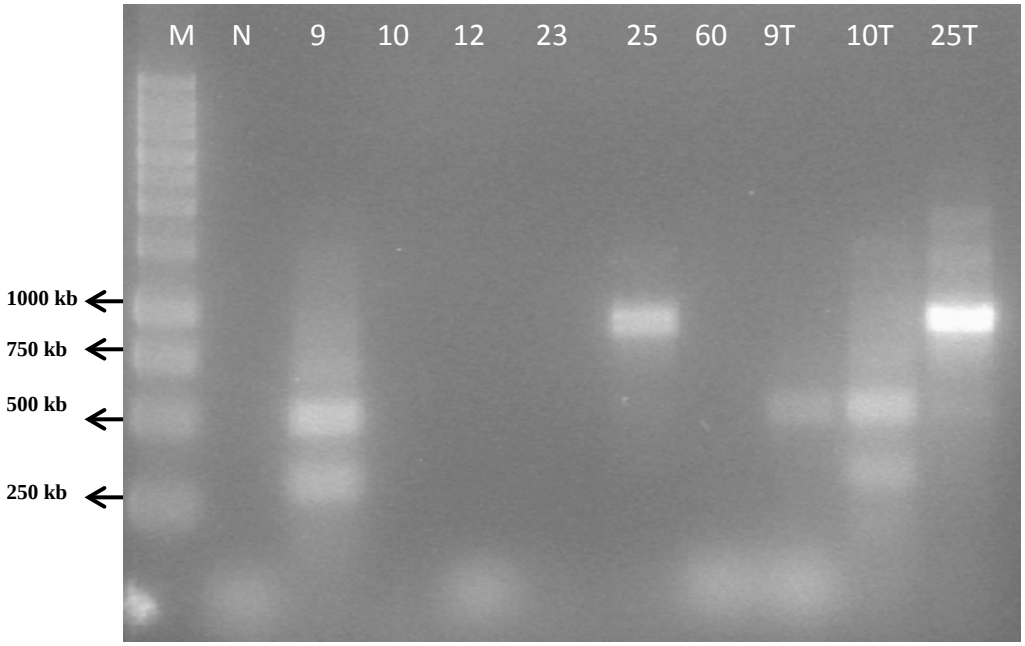


Figure 5.13: PCR amplification of integron class 1 positive isolates 25 and 25T (898 bp). Lanes M: 1 Kb marker. Lane N: negative control (free DNA). The isolate and transconjugant numbers are shown at the top of each lane

PCR product was sequenced giving GenBank No. KC493654.1. The later result confirms the presence of class 1 integron integrase (intI1). Figure 5.14 shows the aligment of sequences of the positive product with Integron class 1.

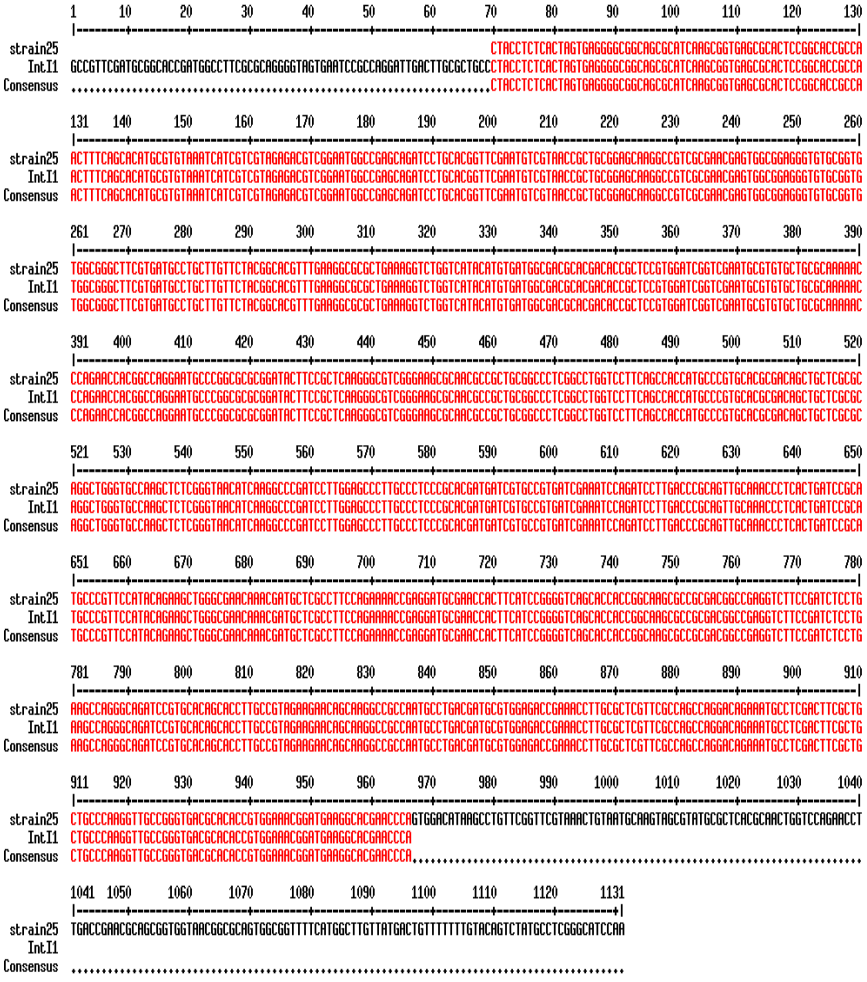


Figure 5.14: IntI1 sequences in isolate 25 are aligning with IntI1. Areas in red are identical

5.8. Discussion

Five strains of *Escherichia coli* and one *Klebsiella pneumoniae* harboured the *bla*CTX-M-15 gene. Two of the *Escherichia coli* strains appeared clonal by PFGE but there was no distinct relationship between these and the other strains. The *bla*CTX-M-15 gene was transferable to *E. coli* J62-2 from three of the strains tested.

Different lengths of truncated IS*Ecp1* (between 171 bp and 544 bp), flanked by IS*26* were detected in all isolates of containing the *bla*CTX-M-15 gene. Sequencing analysis indicated that in all my isolates there was a typical promoter region (-10 TACAAT) and (-35 TTGAA) within the 3' non-coding terminus of IS*Ecp1* which has a crucial role in the increasing expression of *bla*CTX-M-15 (Poirel, Gniadkowski, and Nordmann, 2002; Karim *et al*, 2000; Dhanji *et al*, 2011).

Various *bla*CTX-M-15 genetic environments have been found among *Escherichia coli* isolated from samples obtained from overseas UK travellers, however the majority carried an intact copy of IS*Ecp1* situated 48 bp upstream of *bla*CTX-M-15. This backbone has also been reported in isolates from Canada, Italy, France, Spain, and the UK (Canton and Coque, 2006; Lovally *et al*, 2006; Boyd *et al*, 2004).

My findings correlate with other reports, from Turkey and Germany, that indicated that the *bla*CTX-M-15 genes were located in IncFIA and IncFIB replicons, (Gonullu *et al*, 2008; Mshana *et al*, 2009). However, the IncFII replicons were the most common

associated with *bla*CTX-M-15 genes in Canada, Spain, France, and the UK (Boyd *et al*, 2004; Hopkins *et al*, 2006; Karisik *et al*, 2006; Lavollay *et al*, 2006; Novais *et al*, 2007). My findings in plasmids size in this study indicated that the *bla*CTX-M-15 genes were transferred by large plasmid in most isolates (Figure 5.6) (Table 5.1). The transferred plasmids indicate that the *bla*CTX-M-15 genes migrate in large conjugative plasmids which are well adapted and constantly exchange by lateral gene transfer between our collected isolates in this study.

All isolates producing CTX-M ESBLs investigated in this study were recovered from area in London where there is a large sub-continent population. This may explain the reason for the existence of *bla*CTX-M-15, though it should be noted that the proportion is relatively small. We cannot exclude the possibility that some patients entered hospital already carrying ESBL positive strains, which would explain the diversity. On the other hand, the patients could have caught the strains from the hospital settings, though in this case a greater degree of similarity would be expected.

The connection between enzyme type and location reflects the prevalence patterns for certain CTX-M β-lactamase types (Celenza *et al*, 2006; Mohamed Al-Agamy, El-Din Ashour, and Wiegand, 2006). Recent studies from Sweden and Canada have revealed international travel to be an important risk factor for colonization of gut and community-acquired infections with *Escherichia coli* producing CTX-M ESBLs, reflecting the area or the country visited. For example, the CTX-M-15 β-lactamase is linked to Europe and India, CTX-M-9 and CTX-M-14 β-lactamases are linked with

the Far East and the CTX-M-2 β-lactamases has been linked to South America (Pitout *et al*, 2009; Tham *et al*, 2010).

The results in this study correlates with previous studies that the CTX-M-15 β-lactamase is the predominant member of the group (Novais *et al*, 2007; Nicolas-Chanoine *et al*, 2008; Pitout, 2010; Peirano and Pitout, 2010; Woodford *et al*, 2006; Coelho *et al*, 2010) and confirming that it is the most prevalent member of ESBLs in the UK is *bla*$_{\text{CTX-M-15}}$ gene (Mushtaq *et al*, 2003; Woodford *et al*, 2004). Findings in this study suggest an overall similarity, though with crucial differences, of the genetic arrangement upstream of the *bla*$_{\text{CTX-M-15}}$ gene among all the six isolates. This study suggesst that there possibly was a clone, exemplified by isolates 9 and 10 and the *bla*$_{\text{CTX-M-15}}$ gene was located on different plasmids providing the opportunity to spread the gene among different strains. This suggests that within this population there was diversity both amongst the isolates and the plasmids carrying the *bla*$_{\text{CTX-M-15}}$ gene.

The genetic context of all six isolates in this study was previously described to be the "international *bla*$_{\text{CTX-M-15}}$ genetic environment" (Diestra *et al.*, 2009). This genetic environment was not specific for *Escherichia coli* strains only as it was also found in the strain of *Klebsiella pneumoniae*. Common structures of the international genetic environment of *bla*$_{\text{CTX-M-15}}$ gene are; the "W" region, a disrupted IS*Ecp1* sequence involved promoters which increase expression of the downstream *bla*$_{\text{CTX-M-15}}$ gene, and the existence of orf477 downstream of the *bla*$_{\text{CTX-M-15}}$ gene (Dhanji *et al.*, 2011).

The spread of *bla*$_{\text{CTX-M-15}}$ gene is probably associated to the presence of the insertion sequence, IS*Ecp1*. A strong association between the *bla*$_{\text{CTX-M-15}}$ gene and IS*Ecp1* has been described in literature (Poirel *et al*, 2003; Sonnevend *et al*, 2006; Naseer *et al*, 2009; Ma *et al*, 2009). Furthermore, *bla*$_{\text{CTX-M-15}}$ gene acquisition has often been reported to be mediated by IS*Ecp1* (Yu *et al*, 2011). Indeed insertion sequences have been shown to capture, mobilize, transpose and express of different *bla*$_{\text{CTX-M}}$ genes (Sonnevend *et al*, 2006, Sun *et al*, 2010, Zong *et al*, 2010). IS*Ecp1* was present sequences of all *bla*$_{\text{CTX-M}}$ genes in this study, but with variable degrees of truncation (Figure 5.8). Similar variations have been described in previous studies (Kiratisin *et al*, 2007, Novais *et al*, 2007; Dhanji *et al*, 2011) but not with strain found in such close proximity, which suggests that the plasmids from the strains from St George's hospital are not so closely related. Interestingly, a similar study in strains from Scotland showed far less variation in the truncation of IS*Ecp1* (Dimude & Amyes, 2012). Further indicator of mobilization is the presence of sequences of orf477 (Zong *et al*, 2010). This result was seen downstream of *bla*$_{\text{CTX-M-15}}$ gene in all six isolates in this study.

IS*Ecp1* is not limited to the capture and mobilization of *bla*$_{\text{CTX-M-15}}$ genes; it also enhancing the expression of the downstream *bla*$_{\text{CTX-M-15}}$ gene (Dutour *et al*, 2002; Eckert *et al*, 2006; Ma *et al*, 2011; Peirano and Pitout, 2010). A study by Ma *et al* (2011) revealed that the truncation of IS*Ecp1* adjacent to the *bla*$_{\text{CTX-M-15}}$ gene affects the expression of the gene (Ma *et al*, 2011). This is because the –35 and –10 promoter

sequences reported by Poirel *et al* (2003) ‘’TTGACA’’ and ‘’TAAACT’’ were also detected in the upstream sequences of *bla*$_{\text{CTX-M-15}}$ of all six isolates in this study.

Further analysis, the closest promoter sequences detected in the upstream of *bla*$_{\text{CTX-M-15}}$ was detected to be residing in the “W” sequences. The promoter sequence reported by Dhanji (2011) “TTCAG” was detected in the “W” region of the six isolates in this study. The role of the conserved spacer “W” and explanation of its present upstream *bla*$_{\text{CTX-M-15}}$ remains unclear.

Further investigation of the region of “W” on our six isolates was revealed that it did not only harbour promoter required for the downstream *bla*$_{\text{CTX-M-15}}$ genes expression, but also it had the sequence of Shine-Dalgamo ribosomal binding “AAGGAA” adjacent to the start codon the downstream *bla*$_{\text{CTX-M}}$ gene (Shine and Dalgarno, 1974). The role of these sequences is associated with protein translation. They recruit ribosomes to mRNA for the induction of protein synthesis by aligning them with the start codon (ATG) of the downstream gene. The later sequences were detected in all of the “w” sequences upstream of all *bla*$_{\text{CTX-M-15}}$ genes in this work.

Class 1 integrons are located on plasmids and transposons, create the majority of the integrons detected in the clinical isolates and are related to the multidrug resistance observed in the hospital environment (Martinez-Freijo *et al*, 1998). The presence of gene cassettes as a component of Integron class 1 confers resistance to sulphonamide (cotrimoxazole). Class 1 integrons commonly include an additional resistance gene,

called *sulI*, in the 3' conserved segment, downstream of the gene cassettes (Hall, 2002).

5.9. Conclusion

All our isolates harboured the international *bla*$_{\text{CTX-M-15}}$ genetic environment. This arrangement shows truncated copy of IS*Ecp1* situated 48 bp upstream of *bla*$_{\text{CTX-M-15}}$. Environments that included truncated IS*Ecp1* elements all included the (–35 TTGAA) (–10 TACAAT) promoter within the 3′ non-coding segment involved in the transcriptional of *bla*$_{\text{CTX-M-15}}$. A (–35 TTGACA) and (–10 TAAACT) promoter sequences were also detected in the upstream sequences of *bla*$_{\text{CTX-M-15}}$ of all six isolates in this study. All these finding suggest that as long the increased number of promoters within upstream of *bla*$_{\text{CTX-M-15}}$ gene, the high MICs showed in such isolates. Our study also suggests the impact of travellers on presence and spread of specific genetic arrangement of *bla*$_{\text{CTX-M-15}}$ gene. In addition, this study shows a link between multi drug resistance and the presence of integron in *Escherichia coli*.

Chapter – 6: Conclusions

6. Conclusion

Aim 1. In this study, the detection of ESBLs producing isolates was done by two methods; the double agar dilution method to determine the MICs for three cephalosporins (cefotaxime, ceftazidime, and ceftriaxone) and by the double disc diffusion method with cefotaxime, ceftazidime, and clavulanic acid. The spread of cefotaxime resistance in St George's London hospital was concerning. For further detection and confirmation of the ESBLs producing isolates, PCR was used. The results revealed that the CTX-M-ESBL-producing were responsible for cephalosporin resistance in five isolates of *E. coli* and one isolate of *K. pneumoniae*.

Aim 2. To fulfil the second aim of this work, the MICs were carried out using different classes of antibiotics. The determination of MIC was determined by the double agar dilution method explained by BSAC. Interestingly, co-resistance to fluoroquinolones and aminoglycosides was detected in all ESBLs producing isolates. In addition, this work found that all ESBLs producing isolates in both strains were detected to be susceptible to carbapenems.

Aim 3. PFGE technique was used to determine the relatedness among the CTX-M-producer isolates. This technique was used to study the clonality of these isolates. However, the results of PFGE indicated the genotypic similarity in only two of the six isolates. Therefore, the spread of CTX-ESBL enzymes was mediated by mobile genetic structures.

Plasmid characterization can be detected by S1 nuclease digestion. This technique following PFGE method but using enzymes called S1 nuclease to convert supercoiled plasmids into linear molecules. This study indicated the presence of, at least, one plasmid for each isolates ranging from 78 kb to 152 kb. After extraction and amplification by PCR, plasmids showed replicon type of IncF1A and IncF1B in most of the isolates, suggesting the presence of certain plasmids moving from bacterium to bacterium within the hospital environment. In addition, *bla*$_{\text{CTX-M-15}}$ genes were detected on most plasmids found in these isolates.

Aim 4. With further PCR amplification and sequencing, this project confirmed that *bla*$_{\text{CTX-M-15}}$ is the most prevalent enzyme in St George's London hospital and its gene product the causative enzyme of cephalosporin resistance. Moreover, the results of this study correlate with previous studies from the UK describing CTX-M-15 to be the commonest ESBL present in the UK.

Aim 5. Mobile genetic structures were the main factors leading to increased spread and acquisition of resistance genes. This study focused on studying the genetic environments of *bla*$_{\text{CTX-M-15}}$ and possible genetic elements associated with its spread. Gene walking PCR, plasmid sizing, and conjugation studies assessed this purpose. The results of gene walking PCR showed that IS*Ecp1*was the commonest element of the upstream arrangement of all reported *bla*$_{\text{CTX-M-15}}$. This confirms that IS*Ecp1*

could be the main factor for the increased spread of *bla*$_{\text{CTX-M-15}}$ and responsible for horizontal genetic transfer.

This project shows the molecular diversity associated with the spread of *bla*$_{\text{CTX-M-15}}$ in a major teaching hospital in London. This diversity was observed by different lengths of IS*Ecp1* upstream of *bla*$_{\text{CTX-M-15}}$ genes. In addition, diversity of this gene and its dissemination was shown by different phylogenetic group (A & B2) among the studied isolates. Moreover, the diversity was also represented by the carriage of this gene by both IncFIA and IncFIB plasmids in most of the isolates; it is uncommon that these together carry *bla*$_{\text{CTX-M-15}}$ genes.

On the other hand, the genetic arrangement of *bla*$_{\text{CTX-M-15}}$ genes showed orf477 downstream of *bla*$_{\text{CTX-M-15}}$ in all CTX-M-15 producers. In addition, a typical promoter region (-10 TACAAT) and (-35 TTGAA) was found within the IS*Ecp1* of CTX-M-15 producers which has a crucial role in the increasing expression of *bla*$_{\text{CTX-M-15}}$. Furthermore, the "W" region was 48 bp distant upstream between *bla*$_{\text{CTX-M-15}}$ and IS*Ecp1* in all my isolates in line with other studies.

7. References

7. References

Abraham, E.P. and Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. Nature 146, 837.

Abraham, E.P. Newton, G.G.F. (1961). The structure of cephalosporin C. Biochem J.; 79:377–93.

Abraham, E.P. Baker W. Boon, W.R. Calam, C.T. Carrington, H.C. Florey, H.W. Freeman, G.G. Robison, R. and Sanders, A.G. (1949). Chemistry of penicillin. Edited by Clark H.T. University Press, Princeton.

Alba, J. Ishii, Y. Thomson, K. Moland Ellen, S. and Yamaguchi, K. (2005). Kinetics study of KPC-3, a plasmid-encoded class A carbapenem-hydrolyzing β-lactamase. Antimicrob. Agents Chemother. 49**:**4760–4762.

Albertini, M.T. Benoit, C. Berardi, L. Berrouane, Y. Boisivon, A. Cahen, P. Cattoen, C. Costa, Y. Darchis, P. Delière, E. Demontrond, D. Eb, F. Golliot, F. Grise, G. Harel, A. Koeck, J.L. Lepennec, M.P. Malbrunot, C. Marcollin, M. Maugat, S. Nouvellon, M. Pangon, B. Ricouart, S. Roussel-Delvallez, M. Vachée, A. Carbonne, A. Marty, L. and Jarlier, V. (2002). "Surveillance of methicillin-resistant Staphylococcus aureus (MRSA) and Enterobacteriaceae producing extended-spectrum *β*-lactamase (ESBLE) inNorthern France: a five-year multicentre Incidence study," J. Hosp. Infect. 52 : 107–113.

Alobwede, I. M'Zali, F.H. Livermore, D.M. Heritage, J. Todd, N. and Hawkey, P.M. (2003). CTX-M extended-spectrum β-lactamase arrives in the UK. J. Antimicrob Chemother. 51**:**470-471.

Ambler, R.P. (1980). The structure of β-lactamases. Philos Trans R SocLond B Biol Sci; 289 :321-331.

Ambler, R.P. Coulson, A. F. W. Frere, J. M. Ghuysen, J. M. and Joris, B. Forsman, M. Levesque, R. C. Tiraby, G. and Waley, S. G. (1991). A standard numbering scheme for the class A β-lactamases. Biochem. J. 276**:**269–270.

Amyes, S.G.B. and Smith, J.T. (1974). R-factor trimethoprim resistance mechanism: an insusceptible target site. Biochem Biophys Res Commun. 1974 58: 412–418.

Amyes, S. G. B. (2010). Antibacterial Chemotherapy. 1st Ed. Oxford university press. Oxford, UK.

Anderson, E.S. Threlfall, E.J. Carr, J.M. Mcconnell, M.M. Smith, H.R. (1977). Clonal distribution of resistance plasmid-carrying Salmonella typhimurium, mainly in the Middle East. Journal of Hygiene, 79, 425-448.

Andrews, J.M. (2010). For the BSAC working party on susceptibility testing. BSAC standardised disc susceptibility testing method. J Antimicrob Chemother, 48:5-16.

Andrews, J. M. (2012). BSAC Methods for Antimicrobial susceptibility testing version 10.2.5-91.

Arthur, M. and Courvalin, P. (1993). Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob.Agents Chemother. 37:1563-1571.

Aubron, C. Poirel, L. Ash, J. Ronald, and Nordmann, P. (2005). Carbapenemase-producing *Enterobacteriaceae,* U.S. rivers. Emerg. Infect. Dis. 11**:** 260–264.

Babini, G.S. and Livermore, D.M. (2000). Are SHV β-lactamases universal in *Klebsiella pneumoniae*? Antimicrob. Agents Chemother. 44**:**2230.

Bado, I. Cordeiro, N.F. Robino, L. García-Fulgueiras,V. Seija,V. Bazet, C. Gutkind, G. Ayala, J.A. and Vignoli, R. (2010). Detection of class1 and 2 integrons, extended-spectrum β-lactamases and qnr alleles in enter- obacterial

isolates from the digestive tract of Intensive Care Unit in patients. Int. J. Antimicrob. Agents 36: 453–458.

Bae, I. K. Lee, Y. H. Jeong, H. J. Hong, S.G. Lee, S.H., and Jeong, S.H. (2008). A novel bla (CTX-M- 14) gene harboring complex class 1 integron with an In4-like backbone structure from a clinical isolate of *Escherichia coli*. *Diagn.Microbiol. Infect.Dis.* 62: 340–342.

Bagge, N. Ciofu, O. Hentzer, M. Campbell, J. I. Givskov, M. and Hoiby, N. (2002). Constitutive high expression of chromosomal *β*-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS*1669*) located in *ampD*. Antimicrob. Agents Chemother. 46**:** 3406–3411.

Ball, P.R. Shales, S.W. and Chopra, I. (1980). Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun. 93:74–81.

Bambeke, V. F., Balzi, E. and Tulkens, P. M. (2000). Antibiotic efflux pumps. Biochemical Pharmacology 60: 457–70.

Baquero, M.R. Bouzon, M. Quintela, J. C. Ayala, J. A. and Moreno, F. (1996). *dacD*, an *Escherichia coli* gene encoding a novel penicillin-binding protein (PBP6b) with DD-carboxypeptidase activity. J. Bacteriol. 178:7106–7111.

Baraniak, A. Fiett, J. Sulikowska, A. Hryniewicz, W. and Gniadkowski, M. (2002). Countrywide spread of CTX-M-3 extended-spectrum β-lactamase-producing microorganisms of the family *Enterobacteriaceae* in Poland. Antimicrob. Agents Chemother. 46**:**151-159.

Barthe´le´my, M., J. Pe´duzzi, H. Bernard, C. Tancre`de, and Labia, R. (1992).

Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca.* Biochim. Biophys. Acta 1122:15–22.

Barton, B.M. Harding, G.P. and Zuccarelli, A.J. (1995). A general method for detecting and sizing large plasmids. Anal Biochem 226: 235–240.

Baquero, F. (2004). From pieces to patterns: evolutionary engineering in bacterial pathogens. Nat.Rev.Microbiol. 2: 510–518.

Barlow, M. Reik, R.A. Jacobs, S.D. Medina, M. Meyer, Jr, M.P. McGowan J.E. Tenover, F.C. (2008). High rate of mobilization for blaCTX-Ms. Emerging Infect. Dis.14: 423–428.

Barthélémy, M. Peduzzi, J. Ben Yaghlane, H. & Labia, R. (1988). Single amino acid substitution between SHV-1β-lactamase and cefotaxime-hydrolysing SHV-2 enzyme. FEBS Letters 231, 217–20.

Baudry, P.J. Mataseje, L. Zhanel, G.G. Hoban, D.J. and Mulvey, M.R. (2009). Characterization of plasmids encoding CMY-2 AmpC β-lactamases from *Escherichia coli* in Canadian intensive care units. Diagn.Microbiol.Infect.Dis. 65: 379–383.

Bauernfeind, A. Grimm, H. and Schweighart, S. (1990). A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection 18: 294–298.

Behrens, O.K. Corse, J. Huff, D.E. Jones, R.G. and Soper, Q.F. (1948). Whitehead CW. Biosynthesis of penicillins III. Preparation and evaluation of precursors of new penicillins. J Biol Chem.; 175:771–92.

Bonnet, R. (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob.Agents Chemother.*, 48: 1–14.

Bonnet, R. pitout, C. Sampaio, J.L. M. Chanal, C. Sirot, D. Labia, R. De Champs, C. Labia, R. and Sirot, J. (2001). Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp240Gly. Antimicrob. Agents Chemother. 45**:**2269–2275.

Bonnet, R. Recule, C. Baraduc, R. Chanal, C. Sirot, D. De Champs, C. and Sirot, J. (2003). Effect of D240G substitution in a novel ESBL CTX-M-27. J. Antimicrob. Chemother. 52**:**29-35.

Bonnet, R. Sampaio, J.L. Chanal, C. Sirot, D. De Champs, C. Viallard, J.L. Labia, R. and Sirot, J. (2000). A novel class A extended-spectrum β-lactamase (BES-1) in *Serratia marcescens* isolated in Brazil. Antimicrob. Agents Chemother. 44:3061–3068.

Botta, G.A. and Park, J.T. (1981). Evidence for involvement of penicillinbinding protein 3 in murein synthesis during septation but not during cell elongation. J. Bacteriol. 145:333–340.

Boyd, D. A. S. Tyler, S. Christianson, A. McGeer, M.P. Muller, B.M. Willey, E. Bryce, M. Gardam, Nordmann, P. and Mulvey, M.R. (2004). Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTXM-15 extended-spectrum beta-lactamase involved in an outbreak in longterm care facilities in Toronto, Canada. Antimicrob. Agents Chemother. 48:3758–3764.

Bradford, P.A. Yang, Y. Sahm, D. Grope, I. Gardovska, D. and Storch, G. (1998). CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob. Agents Chemother. 42:1980–1984.

Bradford, P.A. (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 14:933-51.

Bradford, P. A. Urban, C. Mariano, N. Projan, S. J. Rahal, J. J. and Bush, K. (1997). Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase and the loss of an outer membrane protein. Antimicrob. Agents Chemother. 41**:** 563–569.

Branger, **C**. **Zamfir, O. Geoffroy, S. Laurans, G. Arlet, G. Thien, H.V. Gouriou, S. and Picard, B. (2005).** Denamur, E. Genetic background of *Escherichia coli* and extended-spectrum β-lactamase type. Emerg Infect Dis; 11:54–61.

Bratu, S. D. Landman, D. Haag, R. Rocco, R. Eramo, A. Alam, M. and Quale, J. (2005). Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* inNew York City. Arch. Intern. Med. 165:1430–1435.

Bratu, S. Landman, D. Alam, M. Tolentino, E. and Quale, J. (2005b). Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter* spp. From Brooklyn, New York. Antimicrob. Agents Chemother. 49**:**776–778.

Brenwald, N.P. Jevons, G. Andrews, J.M. Xiong, J.H. Hawkey, P.M. and Wise, R. (2003). An outbreak of a CTX-M-type β-lactamase-producing *Klebsiella pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum β-lactamases. J. Antimicrob. Chemother. 51**:**195-196.

Brooks, G. Butel, S. Morse, S. (2007). Enteric Gram-negative rods (Enterobacteriacae). In: Jawetz, Melnick & Adelberg's Medical Microbiology. 24th Ed, McGraw-Hill Medical. New York, USA.

Broome-Smith, J.K. (1985). Construction of a mutant of *Escherichia coli* that has deletions of both the penicillin-binding protein 5 and 6 genes. J. Gen. Microbiol. 131:2115–2118.

Bush, K. (2008). "Extended-spectrum *β*-lactamases in North America, 1987-2006," Clinical Microbiology and Infection. 14**:**134-143.

Bush, K. Jacoby, G.A. and Medeiros, A.A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 39: 1211-1233.

Bush, K. and Jacoby, G.A. (2010). Updatedfunctionalclassification of β-lactamases. Antimicrob.Agents Chemother. 54: 969–976.

Bushman, F. (2002). Lateral DNA transfer. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Buynak, J.D. (2006). Understanding the longevity of the β-lactam antibiotics and of antibiotic/β-lactamase inhibitor combinations. Biochem. Pharmacol., 71:930–940.

Cambray, G. Guerout, A.M. Mazel, D. (2010). Integrons. Annu. Rev. Genet. 44:141–166.

Cantón, R. (2008). "Epidemiologyand evolutionof β-lactamases,"in Evolutionary Biology of Bacterial and FungalPathogens, edsF.Baquero, C. Nombela, G.H. Casslel,andJ. A. Gutierrez-Fuentes (Washington: ASM Press), 249–270.

Canto´ n R. (2009) Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin Microbiol Infect, 15(Suppl. 1):20-25.

Cantón, R. González-Alba J.M, and Galán, J.C. (2012). CTX-M enzymes: origin and diffusion. Front Microbiol 3:110.

Canton, R. Coque, T.M. (2006). The CTX-M β-lactamase pandemic. Curr. Opin.Microbiol. 9, 466–475.

Canton, R. & Morosini, M.I. (2011): Emergence and spread of antibiotic resistance following exposure to antibiotics. FEMS Microbiol Rev, 35:977-991.

Cantón, R. and Ruiz-Garbajosa, P. (2011). Co-resistance:an opportunity for the bacteria and resistance genes. Curr.Opin.Pharmacol. 11: 477–485.

Cantón, R**. Novais, A. Valverde, A. Machado, E. Peixe, L. Baquero, F. and Coque, T. M. (2008).** Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. Clin Microbiol Infect. 14:144-53.

Cao, **V. Lambert, T. Nhu, D.Q. Loan, H.K. Hoang, N.K. Arlet, G. Courvalin, P. (2002).** Distribution of extended-spectrum β-lactamases in clinical isolates of Enterobacteriaceae in Vietnam. Antimicrob. Agents Chemother. 46: 3739–3743.

Carattoli, A. Bertini, A. Villa, L. Falbo, V. Hopkins, K.L. and Threlfall, E.J. (2005). Identification of plasmids by PCR-based replicon typing. J Microbiol Methods, 63:219-28.

Carattoli, A. García-Ferna´ndez, A. Varesi, P. Fortini, D. Gerardi, S. Penni, A. Mancini,C. and Giordano, A. (2008). Molecular epidemiology of *Escherichia coli* producing extended-spectrum β-lactamases isolated in Rome, Ital. J. Clin. Microbiol. 46:103–108.

Carattoli A. (2009). Resistance plasmid families in Enterobacteriaceae. Antimicrob. Agents Chemother. 53: 2227–2238.

Carattoli, A. (2011). Plasmids in Gram negatives: molecular typing of resistance plasmids. Int.J.Med.Microbiol. 301: 654–658.

Castanheira, M. Mendes, R.E. Rhomberg, P.R. and Jones, R.N. (2008). Rapid emergence of *bla*CTX-M among Enterobacteriaceae in U.S. MedicalCenters: molecular evaluation from the MYSTIC Program, Microb. DrugResist. 14: 211–216.

Celenza, G. Pellegrini, C. Caccamo, M. Segatore, M. Amicosante, G. and Perilli, M. (2006). Spread of *bla*CTX-M-type and *bla*PER-2 β-lactamase genes in clinical isolates from Bolivian hospitals. J Antimicrob Chemother; 57: 975–8.

Chain, E. (1979). The early years of the penicillin discovery. Trends Pharmacol Sci.; 1:6–11.

Chain, E. Florey, H.W. Gardner, A.D. Heatley, N.G. Jennings, M.A. Orr-Ewing, J. and Sanders, A.G. (1940). Penicillin as a chemotherapeutic agent. Lancet 2: 226–228.

Chanawong, A. M'Zali, F.H. Heritage, J. Lulitanond, A. and Hawkey, P.M. (2001). SHV-12, SHV-5, SHV-2a and VEB-1extended-spectrum β-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. J. Antimicrob. Chemother. 48**:** 839–852.

Clermont, O. Bonacorsi, S. and Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol; 66: 4555–4558.

Coque, T. M. Baquero, F. and Canton, R. (2008). Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe, Euro Surveillance. 13, no. 47.

Correia, **M**. **Boavida, F. Grosso, F. Salgado, M.J. Lito, L.M. Cristino, J.M. Mendo, S. and Duarte, A. (2003).** Molecular characterization of a new class 3 integron in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 47**:** 2838–2843.

Corvec, S. Caroff, N. Espaze, E. Giraudeau, C. Drugeon, H. and Reynaud, A. (2003). AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. J. Antimicrob. Chemother. 52**:** 629–635.

Cottell, J.L. Webber, M.A. Coldham, N.G. Taylor, D.L. CerdeñoTárraga, A.M. Hauser, H. Thomson, N.R. Woodward, M.J. and Piddock, L.J. (2011). Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding *bla*$_{\text{CTX-M-14}}$. Emerging Infect.Dis. 17: 645–652.

Coudron, P.E. Hanson, N.D. and Climo, M.W. (2003). Occurrence of extended-spectrum and AmpC *β*-lactamases in bloodstream isolates of *Klebsiella pneumoniae*: isolates harbour plasmid-mediated FOX–5 and ACT–1 AmpC β-lactamases. J. Clin. Microbiol. 41**:** 772–777.

Cullik, A. Pfeifer, Y. Prager, R. von Baum, H. and Witte, W.A. (2010). Novel IS26 structure surrounds *bla*$_{\text{CTX-M}}$ genes in different plasmids from German clinical isolates of *Escherichia coli*.J Med Microbiol. 59:580–7.

Datta, N. Dacey, S. Hughes, V. Knight, S. Richards, H. Williams, G. Casewell, M. and Shannon, K.P. (1980). Distribution of genes for trimethoprim and gentamicin resistance in bacteria and their plasmids in a general hospital. J. Gen. Microbiol. 118: 495–508.

Datta, N. and Hedges, R.W. (1971). Compatibility groups among fi^{-} R factors. Nature 234**:**222-223.

Datta, N. and Kontomichalou, P. (1965). Penicillinase synthesis controlled by infectious R Factors in Enterobacteriaceae. Nature 208: 239-244.

Davin-Régli, A. and Pagès, J.M. (2006). In Antimicrobial Resistance in Bacteria (ed. Zmabiles-Cuevos, C. F.) 55–75 (Horizon Biosciences, Norfolk, 2006).

Desjardins P., Picard B., Kaltenböck B., Elion J., Denamur E. (1995). Sex in Escherichia coli does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment length polymorphism. J. Mol. Evol. 41:440–448.

Dhanji, H. Patel, R. Wall, R. Doumith, M. Patel, B. Hope, R. Livermore, D.M. and Woodford, N. (2011). Variation in the genetic environments of bla(CTX-M-15) in *Escherichia coli* from the faeces of travellers returning to theUnited Kingdom. J. Antimicrob.Chemother. 66, 1005–1012.

Dhillon, R.H. and Clark, J. (2012). ESBLs: a clear and present danger. Crit Care Res Pract. 62:5170.

Diestra, K. Juan, C. Curiao, T. Moya, B. Miro, E. Oteo, J. Coque, T.M. Perez-Vazquez, M. Campos, J. Canton, R. Oliver, A. and Navarro, F. (2009). Characterization of plasmids encoding *bla*ESBL and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. J Antimicrob Chemother. 63: 60-66.

Dimude, J. and Amyes, S.G.B. (2012). Characterization of IncFIA, IncFIB, IncF11 and IncN Plasmids Carrying CTX-M-15, TEM-1, OXA-1 β-lactamases and *acc(6')-IB-cr* gene from *Escherichia coli*. Clin. Microbiol. Infect. 18: 734.

Doi, Y. Park, Y.S. Rivera, J.I. Adams-Haduch, J.M. Hingwe, A. Sordillo, E.M. Lewis, J.S. 2nd. Howard, W.J. Johnson, L.E. Polsky, B. Jorgensen, J.H. Richter, S.S. Shutt, K.A. and Paterson, D.L. (2012). Community-Associated Extended-Spectrum-β-Lactamase (ESBL)-Producing *Escherichia coli* Infection in the United States, Clin Infect Dis, 56: 641-8.

Du Bois, S.K. Marriott, M.S. and Amyes, S.G.B. (1995). TEM and SHV-derived extended-spectrum -lactamases: relationship between selection, structure and function. Journal of Antimicrobial Chemotherapy 35: 7–22.

Duriez, P. Clermont, O. Bonacorsi, S. Bingen, E. Chaventre, A. Elion, J. Picard, and B. Denamur, E. (2001). Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology; 147:1671-1676.

Dutour, C. Bonnet, R. Marchandin, H. Boyer, M. Chanal, C. Sirot, D. and Sirot, J. (2002). CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from *Enterobacteriaceae* isolated in France. Antimicrob. Agents Chemother. 46**:**534-537.

Eckert, C. Gautier, V. and Arlet, G. (2006). DNA sequence analysis of the genetic environment of various blaCTX-M genes. J. Antimicrob. Chemother. 57: 14–23.

Edelstein, M. Pimkin, M. Palagin, I. Edelstein, I. and Stratchounski, L. (2003). Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing Escherichia coli and *Klebsiella pneumoniae* in Russian hospitals. Antimicrob Agents Chemother; 47:3724-32.

Eisner, A. Fagan, E.J. Feierl, G. Kessler, H.H. Marth, E. Livermore, D.M. and Woodford, N. (2006). Emergence of Enterobacteriaceae isolates producing CTX-M extended-spectrum β-lactamase in Austria. Antimicrob Agents Chemother; 50:785–787.

Ejrnaes, K. Sandvang, D. Lundgren, B. Ferry, S. Holm, S. Monsen, T. Lundholm, R. and Frimodt-Moller, N. (2006). Pulsed field gel electrophoresis typing of *Escherichia coli* strains from samples collected before and after pivmecillinam or placebo treatment of uncomplicated community acquired urinary tract infection in women. J. Clin. Microbiol., 44: 1776-1781.

Evans, B. A. Hamouda, A. and Amyes, S.G.B. (2007). OXA-type β-lactamases in *Acinetobacter baumannii*: emerging from the shadow of the extended-spectrum β-lactamases. Rev.Med.Microbiol. 18:63-72.

Evans, B.A. Hamouda, A. and Amyes, S.G.B. (2013). The rise of carbapenem-resistant *Acinetobacter baumannii*. Current Pharmaceutical Design 19:223-238.

Falagas, M. E. and Karageorgopoulos, D. E. (2009). Extended spectrum β-lactamase-producing organisms.J. Hosp. Infect. 4: 345–354.

Neidhardt F. C., Curtiss III R., Ingraham J. L., Lin E. C. C., Low K. B., Magasanik B., Reznikoff W. S., Riley M., Schaechter M., Umbarger H. E. Falkow S. (1996). The evolution of pathogenicity in Escherichia coli, Shigella, and Salmonella. in Escherichia coli and Salmonella: cellular and molecular biology, eds NeidhardtF. C., Curtiss IIIR., IngrahamJ. L., LinE. C. C., LowK. B., MagasanikB., ReznikoffW. S., RileyM., SchaechterM., UmbargerH. E. (ASM Press, Washington, D.C), 2nd ed. pp 2723–2729.

Fairbrother, R.W. and Taylor, G. (1960). Lancet Aug. 20: 400.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. Br J Exp Pathol X:3-13.

Fisher, J. F. Meroueh, S. O. and Mobashery, S. (2005). Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. Chem Rev 105:395-424.

Fosberry, A.P. Payne, D.J. Lawlor E.J. and Hodgson, J.E. (1994). Cloning and sequence analysis of bla_{BIL-1}, a plasmid- mediated class C β-lactamase gene in *Escherichia coli* BS. Antimicrob.Agents Chemother. 38:1182-1185.

Francia, M.V. Varsaki, A. Garcillan-Barcia M.P. Latorre, A. Drainas, C. (2004). A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. 28: 79–100.

Frere, J.M. Nguyen-DisteÁche, M. Coyette, J. and Joris, B. (1992). Mode of action: interaction with the penicillin-binding proteins. In The Chemistry of β-Lactams. Page, M.I. (ed.). London: Blackie Academic and Professional, 148-197.

Galas, M, Decousser, J.W, Breton, N, Godard, T, Allouch, P.Y, Pina, P. (2008). Nationwide study of the prevalence, characteristics, and molecular epidemiology of extended-spectrum-beta-lactamase-producing Enterobacteriaceae in France. Antimicrob Agents Chemother. 2008;52(2):786-9.

García-Fernández, A. Cloeckaert, A. Bertini, A. Praud, K. Doublet, B. Weill, F.X. and Carattoli, A. (2007). Comparative analysis of IncHI2 plasmids carrying blaCTX-M-2or blaCTX-M-9 from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. Antimicrob.AgentsChemother. 51: 4177–4180.

Garcia-Fernandez, A. Fortini, D. Veldman, K. Mevius, D. and Carattoli, A. (2009). Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. J Antimicrob Chemother, 63:274-81.

Garcia-Rodriguez, J.A. and Jones, R.N. (2002). Antimicrobial resistance in gram-negative isolates from European intensive care units: data from the Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) programme. J.Chemother. 14**:** 25–32.

Gazouli, M. Tzelepi, E. Sidorenko, S.V. and Tzouvelekis, L.S. (1998). Sequence of the gene encoding a plasmid-mediated cefotaxime-hydrolyzing class A β-lactamase (CTX-M-4): involvement of serine 237 in cephalosporin hydrolysis. Antimicrob. Agents Chemother. 42**:**1259–1262.

Germond, J.E. Vogt, V.M. Hirt, B. (1974). Characterization of the single-strand-specific nuclease S1 activity on double-stranded supercoiled polyoma DNA. Eur J Biochem. 1974; 43:591–600.

Ghuysen, J. M. (1988). Bacterial active-site serine penicillin-interactive proteins and domains: mechanism, structure, and evolution. Rev Infect Dis 10: 726-732.

Giakkoupi, P. Tzouvelekis, L.S. Tsakris, A. Loukova, V. Sofianou, D. and Tzelepi, E. (2000). IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *Enterobacter cloacae* clinical strain. Antimicrob. Agents Chemother. 44**:** 2247–2253.

Gierczynski, R. Szych, J. Rastawicki, W. and Jagielski, M. (2003). The molecular characterisation of the extended spectrum β-lactamase (ESBL) producing strain of *Salmonella enterica* serovar Mbandaka isolated in Poland. Acta Microbiol. Pol. 52**:** 183–190.

Gill, M.J. Brenwald, N.P. and Wise, R. (1999). Identification of an efflux pump gene pmrA, associated with fluoroquinolone resistance in Streptococcus pneumoniae. Antimicrobial Agents and Chemotherapy 43: 187–9.

Girlich, D. Poirel, L. Leelaporn, A. Karim, A. Tribuddharat, C. Fennewald, M. and Nordmann, P. (2001). Molecular epidemiology of the integron located VEB-1 extended-spectrum β-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand. J. Clin. Microbiol. 39**:** 175–182.

Gonullu, N. Aktas, Z. Kayacan, C.B. Salcioglu, M. Carattoli, A. Yong, D.E. and Walsh, T.R. (2008). Dissemination of CTX-M-15 β-lactamase genes carried on Inc FI and FII plasmids among clinical isolates of *Escherichia coli* in a university hospital in Istanbul, Turkey. J. Clin. Microbiol. 46**:**1110-1112.

Gniadkowski, M. Schneider, I. Pałucha, A. Jungwirth, R. Mikiewicz, B. Bauernfeind, A. (1998). Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-

hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob. Agents Chemother. 42**:**827–832.

Gonullu, N. Aktas, Z. Kayacan, C.B. Salcioglu, M. Carattoli, A. Yong, D.E. and Walsh, T.R. (2008). Dissemination of CTX-M-15 β-lactamase genes carried on Inc FI and FII plasmids among clinical isolates of **Escherichia coli** in a university hospital in Istanbul, Turkey. J. Clin. Microbiol. 46**:**1110-1112.

Goossens, H. (2001). MYSTIC program: summary of European data from 1997 to 2000. Diagn. Microbiol. Infect. Dis. 41**:** 183–189.

Greenwood, D. Slack, R. and Peutherer, J. (1992). Medical Microbiology: A Guide to Microbial Infections: Pthogenesis, Immunity, Laboratory Diagnosis and Control, 14 edition, Churchil Livingstone.

Guiral, E. Mendez-Arancibia, E. Soto, S.M. Salvador, P. Fàbrega, A. Gascón, J. and Vila, J. (2011). CTX-M-15–producing Enteroaggregative *Escherichia coli* as Cause of Travelers'Diarrhea, Emerging Infectious Diseases; Vol. 17, No. 10.

Gupta, V. (2007). An update on newer β-lactamases. Indian J Med Res 126: 417–427.

Hackel, **M. Badal**, **M. Bouchillon**, **R. Johnson, S. Johnson, B. Hoban, J. Dowzicky, M**. **(2008).** Extended-spectrum β-lactamase production in Europe. In: Abstracts of the 18th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Barcelona, Spain.

Hall, R.M. (2002). Gene cassettes and integrons: moving single genes. In: Syvanen M, Kado CI, eds. Horizontal gene transfer. 2nd ed. San Diego: Academic Press:19–28.

Hall, R.M. and Collis, C.M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Molecular Microbiology 15: 593–600.

Handwerger, S. and Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. Rev. Infect. Dis. 7:368–382.

Hanson, N.D. and Sanders, C.C. (1999). Regulation of inducible AmpC *β*-lactamase expression among Enterobacteriaceae. Curr. Pharm. Des. 5**:** 881–894.

Helfand, M. S. and Bonomo, R. A. (2003). β-lactamases: a survey of protein diversity. Curr. Drug Targets. Infect. Disord. 3: 9–23.

Hibbert-Rogers, L.C. Heritage, J. Gascoyne-Binzi, D.M. Gascoyne-Binzi, P.M. Hawkey, P.M. Todd, N. Lewis, J. and Bailey, C. (1995). Molecular epidemiology of ceftazidime resistant Enterobacteriaceae patients on a paediatric oncology ward. J. Antimicrob. Chemother. 36:65–82.

Hien, T.T. and White, N.J. (1993). Qinghaosu. Lancet; 341:603–608.

Hopkins, K.L. Liebana, E. Villa, L. Batchelor, M. Threlfall, E.J. Carattoli, A. (2006). Replicon typing of plasmids carrying CTX-M or CMY beta-lactamasescirculating among *Salmonella* and *Escherichia coli* isolates. Antimicrob Agents Chemother;50:3203–3206.

Hossain, A. Ferraro, M.J. Pino, R.M. Dew III, R.B. Moland, E.S. Lockhart, T.J. Thomson, K.S. Goering, R.V. and Hanson, N.D. (2004). Plasmid mediated carbapenem-hydrolyzing enzyme KPC-2 in an *Enterobacter* sp. Antimicrob. Agents Chemother. 48**:**4438–4440.

Houvinen, P. Houvinen, S. and Jacoby, G.A. (1988). Sequence of PSE-2 β-lactamase, Antimicrob Agents Chemotherapy, 32: 134-136.

Hrabak, J.J. Empel, M. Gniadkowski, Z. Halbhuber, K. Rebl, and Urbaskova, P. (2008). CTX-M-15-producing *Shigella sonnei* strain from a Czech patient who traveled in Asia. J. Clin. Microbiol. 46:2147–2148.

Hu, W.Y.and Derbyshire, K.M. (1998). Target Choice and Orientation Preference of the Insertion Sequence IS*903*, J.Bacteriol. 180:3039–3048.

Huletsky, A.J. Knox, R. and Levesque, R.C. (1993). Role of Ser-238 and Lys-240 in the hydrolysis of 3rd-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and 3-dimensional modeling. J. Biol. Chem. 268**:**3690–3697.

Ibuka, A.S. Ishii, Y. Galleni, M. Ishiguro, M. Yamaguchi, K. Frere, J.M. Matsuzawa, H. Sakai, H. (2003). Crystal structure of extended-spectrum β-lactamase Toho-1: insights into the molecular mechanism for catalytic reaction and substrate specificity expansion. Biochemistry 42**:** 10634–10643.

Imada, A. Kitano, K. Muroi, M. and Asai, M. (1981). Sulfazecin and isosulfazecin, novel β-lactam antibiotics of bacterial origin. Nature. 289:590–591.

Ishii, Y. Ohno, A. Taguchi, H. Imajo, S. Ishiguro, M. and Matsuzawa, H. (1995). Cloning and sequence of the gene encoding a cefotaxime-hydrolyzingclass A β-lactamase isolated from *Escherichia coli.* Antimicrob. Agents Chemother. 39**:**2269–2275.

Jacoby, G.A. and Munoz-Price, L.S. (2005). The new β-lactamases. New Eng J. Med, 352: 380–391.

Jacoby, G.A. and Sutton, L. (1991). Properties of plasmids responsible for production of extended-spectrum β-lactamases. Antimicrob. Agents Chemother.35**:**164–169.

Jarlier, V. Nicola, M.H. Fournier, G. and Philippon, A. (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 10: 867–878.

Jones, R.N. (1998). Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the Amp C enzymes. Diagn. Microbiol. Infect. Dis. 31**:** 461–466.

Kaatz, G.W. and Seo, S.M. (1995). Inducible Nor A-mediated multidrug resistance in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 39: 2650–2655.

Kaier, K. Frank, U. Hagist, C. Conrad, A. Meyer, E. (2009). The impact of antimicrobial drug consumption and alcohol-based hand rub use on the emergence and spread of extended-spectrum β-lactamase-producing strains: a time-series analysis. J Antimicrob Chemother; 63:609-614.

Karim, A.L. Poirel, S. Nagarajan, and Nordmann, P. (2001). Plasmid mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. FEMS Microbiol. Lett. 201**:**237–241.

Karisik, E. Ellington, M.J. Pike, R. Warren, R.E. Livermore, D.M. and Woodford, N. (2006). Molecular characterization of plasmids encoding CTX-M-15 beta-lactamases from *Escherichia coli* strains in the United Kingdom. J Antimicrob Chemother;58:665–668.

Karisik, E. Ellington, M.J. Livermore, D.M. and Woodford, N. (2008). Virulence factors in *Escherichia coli* with CTX-M-15 and other extended-spectrum β-lactamases in the UK. J Antimicrob Chemother; 61:54-58.

Kariuki, S. J. E. Corkill, G. Revathi, R. Musoke, and Hart, C. A. (2001).

Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob. Agents Chemother. 45**:**2141–2143.

Katsanis, P.G. J. Spargo, M.J. Ferraro, Sutton, L. and Jacoby, G. (1994). Detection of *Klebsiella pneumoniae* and *Escherichia coli* strains producing extended-spectrum β-lactamases. J. Clin. Microbiol. 32: 691-696.

Ke, W. Bethel, C.R. Thomson, J.M. Bonomo, R.A. and vanden Akker, F. (2007). Crystal structure of KPC-2: insights into carbapenemase activity in class A β-lactamases. Biochemistry 46**:**5732–5740.

Kliebe, C. Nies, B.A. Meyer, J.F. Tolxdorff-Neutzling, R.M. & Wiedemann, B. (1985). Evolution of lasmid-coded resistance to broad-spectrum cephalosporins. Antimicrobial Agents and Chemotherapy 28, 302–7.

Kerrn, M.B. Klemmensen, T. Frimodt-Møller, N. and Espersen, F. (2002). Susceptibility of Danish Escherichia coli strains isolated from urinary tract infections and bacteraemia, and distribution of sul genes conferring sulphonamide resistance. J Antimicrob Chemother; 50: 513–6.

Kingston, W. (2000). Antibiotics, invention and innovation. Research Policy, 29: 679-710.

Kiratisin, P. apisarnthanarak, A. Saifon, P. Laesripa, C. Kitphati, R. and Mundy, L.M. (2007). The emergence of a novel ceftazidime-resistant CTX-M extended-spectrum beta-lactamase, CTX-M-55, in both community-onset and hospital-acquired infections in Thailand. Diagn Microbiol Infect Dis, 58:349-55.

Kjerulf A, Hansen DS, Sandvang D, Hansen F, Frimodt-Møller N. (2008). The prevalence of ESBL-producing *E. coli* and *Klebsiella* strains in the Copenhagen area of Denmark. APMIS;116(2):118-24.

Kliebe, C. Nies, B.A. Meyer, J.F. Tolxdorff-Neutzling, R.M. Wiedemann, B. (1985). Evolution of plasmid coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. 28: 302-307.

Knight, R. (1980). The chemotherapy of amoebiasis. J. Antimicrob. Chemother. 6:577–593.

Knothe, H. Shah, P. Krcmery, V. Antal, M. Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection 11: 315–317.

Knott-Hunziker, V. Petursson, S. Waley, S. G. Jaurin, B. and Grundstrom, T. (1982). The acyl-enzyme mechanism of β-lactamase action. The evidence for class C β-lactamases. Biochem J, 207: 315–322.

Kumagai, **Toshio; Tamai, Satoshi; Abe, Takao; Hikida, Muneo. (2002).** Current Medicinal Chemistry Anti-Infective Agents. 1: 1-14.

Labia, R. (1999). Analysis of the bla_{Toho} gene coding for Toho-2 β-lactamase. Antimicrob. Agents Chemother. 43**:**2576-2577.

Lahti, C.J. (1996). Pulsed field gel electrophoresis in the clinical microbiology laboratory, J Clin Lab Anal. 10:326-330.

Lartigue, M.F. Poirel, L, Aubert, D. Nordmann, P. (2006). *In vitro* analysis of IS*Ecp1* B-mediated mobilization of naturally occurring β-lactamase gene *bla*CTX-M of *Kluyvera ascorbata*. Antimicrob Agents Chemother; 50: 1282–1286.

Lartigue, M.F. Poirel, L. and Nordmann, P. (2004). Diversity of genetic environment of $bla_{\text{CTX-M}}$ genes. FEMS Microbiol Lett 234: 201–207.

Laupland, K.B. Church, D.L. Vidakovich, J. Mucenski, M. and Pitout, J.D. (2008). Community-onset extended-spectrum β-lactamase (ESBL) producing Escherichia coli: importance of international travel. J. Infect. 57**:**441–448.

Lecointre, G. Rachdi, L. Darlu, P. Denamur, E. (1998). Escherichia coli molecular phylogeny using the incongruence length difference test. Mol. Biol. Evol. 15:1685–1695.

Lee, S. Yu, J.K. Park, K. Oh, E.J. Kim, S.Y. and Park, Y.J. (2010). Phylogenetic groups and virulence factors in pathogenic and commensal strains of *Escherichia coli* and their association with blaCTX-M. Ann Clin Lab Sci 40:361–367.

Leflon-Guibout, V. Blanco, J. Amaqdouf, K. Mora, A. Guize, L.and Nicolas-Chanoine, M.H. (2008). Absence of CTX-M enzymes but a high prevalence of clones, including clone ST131, among the fecal *Escherichia coli* isolates of healthy subjects living in the Paris area. J. Clin. Microbiol. 46:3900–3905.

Levy, S.B. and McMurry, L. (1974). Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. Biochem Biophy Res Commun. 56: 1060-1068.

Lewis, J.S. Herrera, M. Wickes, B. Patterson, J.E. and Jorgensen, J.H. (2007). First report of the emergence of CTX-M-type extended-spectrum *β*-lactamases (ESBLs) as the predominant ESBL isolated in a U.S. health care system, Antimicrobial Agents and Chemotherapy, 11: 4015–4021.

Li, S. Qu, Y. Hu, D. and Shi, Y.X. (2012). Comparison of extended spectrum β-lactamases producing *Escherichia coli* with non-ESBLs producing *E.coli*: drug-resistance and virulence, World J Emerg Med. 3: 208–212.

Li, X. Z. Livermore, D.M. and Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of Pseudomonas aeruginosa resistance to tetracycline,

chloramphenicol, and norfloxacin. Antimicrobial Agents and Chemotherapy 38: 1732–41.

Lin, J., Michel, L. O. and Zhang, Q. (2002). Cme ABC functions as a multidrug efflux system in Campylobacter jejuni. Antimicrobial Agents and Chemotherapy 46: 2124–31.

Livermore, D.M. (1995). β-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 8:557–584.

Livermore, D.M. Canton, R. and Gniadkowski, M. (2007). CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 59:165–174.

Livermore, D. M. and Hawkey, P. M. (2005). CTX-M: changing the face of ESBLs in the UK. J Antimicrob Chemother, 56: 451–454.

Livermore, D. M. and Williams, J. D. (1996). Mode of action and mechanisms of bacterial resistance. In V. Lorian (Ed.), Antibiotics in Laboratory Medicine, 4th Ed. New York, Williams and Wilkins, Baltimore 502-578.

Livermore, D.M. and Woodford, N. (2006). The β-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol 14: 413-420.

Lomovskaya, O. Warren, M.S. Lee, A. Galazzo, J. Fronko, R. and Lee, M. (2001). Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. Antimicrobial Agents and Chemotherapy 45: 105–116.

Lavollay M, Mamlouk K, Frank T, Akpabie, A. Burghoffer, B. Ben Redjeb, R.S. (2006). Clonal dissemination of a CTX-M-15 β-lactamase-producing *Escherichia coli* strain in the Paris area, Tunis, and Bangui. Antimicrob Agents Chemother.50:2433-2438.

Ma, L. Siu, L.K. and Lu, P.L. (2011). Effect of spacer sequences between *bla*CTX-M and ISEcp1 on blaCTX-M expression. J. Med.Microbiol. 60: 1787–1792.

Mariotte-Boyer, S. Nicolas-Chanoine, M. H. and Labia, R. (1996). A kinetic study of NMC-A β-lactamase, an Ambler class A carbapenemase also hydrolyzing cephamycins. FEMS Microbiol. Lett. 143**:**29–33.

Majiduddin, F.K. and Palzkill, T. (2003). Amino acid sequence requirements at residues 69 and 238 for the SME-1 β-lactamase to confer resistance to β-lactam antibiotics. Antimicrob. Agents Chemother. 47**:**1062–1067.

Majiduddin, F.K., and Palzkill, T. (2005). Amino acid residues that contribute to substrate specificity of class A β-lactamase SME-1. Antimicrob. Agents Chemother. 49**:**3421–3427.

Martinez-Freijo P, Fluit AC, Schmitz F-J, Grek VSC, Verhoef J, Jones ME. Class 1 integrons in gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. J Antimicrob Chemother; 42: 689-96.

Martine´z-Martine´z, L. S. Herna´ndez-Alle´s, S. Albertı´, J. Toma´s, V. Benedi, and G. Jacoby. (1996). In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expandedspectrum cephalosporins. Antimicrob. Agents Chemother. 40:342–348.

Massova, I. and Mobashery, S. (1998). Kinship and diversification of bacterial penicillin-binding proteins and *β*-lactamases, Antimicrob. Agents Chemother; 42:1-17.

Masterton, R.G. and Turner, P.J. (2006). Trends in antimicrobial susceptibility in UK centres: the MYSTIC Programme (1997–2002). Int.J Antimicrob.Agents., 27, 69–72.

Matagne, A. Lamotte-Brasseur, J. and Frere, J.M. (1998). Catalytic properties of class A β-lactamases: efficiency and diversity. Biochem J 330: 581–598.

Mathur, P. Kapil, A. Das, B. and Dhawan, B. (2002). Prevalence of extended spectrum β-lactamase producing gram negative bacteria in a tertiary care hospital. Indian J Med Res; 115:153-57.

Matsumoto, Y.F. Ikeda, T. Kamimura, Y. Yokota, and Mine, Y. (1988). Novel plasmid-mediated β-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrob. Agents Chemother. 32**:**1243–1246.

Matsumoto, Y. and Inoue, M. (1999). Characterization of SFO-1, a plasmid-mediated inducible class A β-lactamase from *Enterobacter cloacae*. Antimicrob. Agents Chemother. 43**:** 307–313.

Matthew, M., Hedges R. W., and J. T. Smith. (1979). Types of β-lactamase determined by plasmids in gram-negative bacteria. J. Bacteriol. 138:657–662.

Medeiros, A. A. (1997). Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. 24 Suppl. 1**:**S19-45.

Medeiros, A.A. and Hare, R.S. (1986). β-Lactamase-mediated resistance to penems and carbapenems amongst *Enterobacteriaceae*, abstr. 116. 26th Intersci.Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC.

Melano, R. Corso, A. Petroni, A. Centron, D. Orman, B. Pereyra, A. Moreno, N; Galas, M. (2003). Multiple antibiotic-resistance mechanisms including a novel

combination of extended-spectrum β-lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina. J. Antimicrob. Chemother. 52**:** 36–42.

McManus, M.C. (1997). Mechanisms of bacterial resistance to antimicrobial agents. Am J Health Syst Pharm., 54: 1420-1433.

Miriagou, V. Tzouvelekis, L.S. Rossiter, S. Tzelepi, E. Angulo, F.J. and Whichard, J.M. (2003). Imipenem resistance in a *Salmonella* clinical strain due to plasmid-mediated class A carbapenemase KPC-2. Antimicrob. Agents Chemother. 47**:**1297–1300.

Mohamed Al-Agamy, M.H. El-Din Ashour, M.S. Wiegand, I. (2006). First description of CTX-M b-lactamase-producing clinical Escherichia coli isolates from Egypt. Int J Antimicrob Agents 27: 545–548.

Moland, E.S. Hanson, N. D. Herrera, V.L. Black, J. A. Lockhart, T. J. Hossain, A. Johnson, J. A. Goering, R.V. and Thomson, K. S. (2003). Plasmid mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J. Antimicrob. Chemother. 51**:**711–714.

Montes J., Cueva J. Ehrlich. (1967). Otten su vida y su obra. Alergia; 15: 29–42.

Mshana SE, Imirzalioglu C, Hossain H, Hain, T. Domann, E. and Chakraborty, T. Conjugative IncFI plasmids carrying CTX-M-15 among Escherichia coli ESBL producing isolates at a University hospital in Germany. BMC Infect Dis;9:97.

Mugnier, P.P. Dubrous, I. Casin, G. Arlet, and Collatz. E. (1996). A TEM-derived extended-spectrumβ-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 40**:**2488–2493.

Mushtaq, S. Woodford, N. Potz, N. and Livermore, D.M. (2003). Detection of CTX-M-15 extended-spectrum β-lactamase in the United Kingdom. J. Antimicrob. Chemother. 52**:**528-529.

Mourey, L. Miyashita, K. Swaren, P. Bulychev, A. Samama, J.P. and Mobashery, S. (1998). Inhibition of the NMC-A β-lactamase by a penicillanic acid derivative and the structural bases for the increase in substrate profile of this antibiotic resistance enzyme. J. Am. Chem. Soc. 120**:**9382–9383.

Naas, T. Mikami, Y. Imai, T. Poirel, L. and Nordmann, P. (2001). Characterization of In53, a class 1 plasmid- and composite transposon-located integron of Escherichia coli which carries an unusual array of gene cassettes.J Bacteriol 183: 235–249.

Naas, T. and Nordmann, P. (1994). Analysis of a carbapenem-hydrolyzing class A β-lactamase from *Enterobacter cloacae* and of its LysR-type regulatory protein. Proc. Natl. Acad. Sci. USA 91**:**7693–7697.

Naas, T. and Nordmann, P. (1999). OXA-type β-lactamases. Curr. Pharm. Des. 5:865-79.

Naas, T. Philippon, L. Poirel, L. Ronco, E. and Nordman, P. (1999). An SHV-derived extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 43**:**1281–1284.

Naas, T. L. Poirel, and Nordmann, P. (2008). Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. 14**:**42–52.

Nadjar, D. Labia, R. Cerceau, C. Bizet, C. Philippon, A. and Arlet, G. (2001). Molecular characterization of chromosomal class C *β*-lactamase and its regulatory gene in *Ochrobactrum anthropi*. Antimicrob. Agents Chemother. 45**:** 2324–2330.

Nagarajan R. Boeck L.D. Gorman M. Hamill R.L. Higgens C.E. and Hoehn M.M. (1971). B-lactam antibiotics from *Streptomyces*. J Am Chem Soc; 93:2308–10.

Naseer, U. Haldorsen, B. Tofteland, S. Hegstad, K. Scheutz, F. Simonsen, G.S. Sundsfjord, A. (2009). Molecular characterization of CTX-M-15-producing clinical isolates of Escherichia coli reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway. APMIS. 177:526-36.

Navarro, F. Mesa, R.J. Miro, L. Gomez, B. Mirelis, and Coll, P. (2007). Evidence for convergent evolution of CTX-M-14 ESBL in *Escherichia coli* and its prevalence. FEMS Microbiol. Lett. 273:120–123.

Nestorovich, E.M. Sugawara, E. Nikaido, H. and Bezrukov, S.M. (2006). *Pseudomonas aeruginosa* porin OprF. Properties of the channel. *J. Biol. Chem.* 281, 16230–16237.

Neu, H.C. (1992). The crisis in antibiotic resistance. Science;257:1064–1073.

Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. 33: 1831–1836.

Nikaido, H. (2000). Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Seminars in Cellular and Developmental Biology 12: 215–33.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67: 593–656.

Nordmann, P. Lartigue, M.F. and Poirel, L. (2008). β-lactam induction of ISEcp1B-mediated mobilization of the naturally occurring bla (CTX- M) β-lactamase gene of *Kluyvera ascorbata*. FEMS Microbiol. Lett. 288: 247–249.

Nordmann, P. Ronco, E. Naas, T. Duport, C. Michel-Briand, Y. and Labia, R. (1993). Characterization of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 37**:**962–969.

Normark, b.h., Normark, S. (2002). Evolution and spread of antibiotic resistance. J Intern Med; 252: 91–106.

Novais, A. Cantón, R. Moreira, R. Peixe, L. Baquero, F. and Coque, T.M. (2007). Emergence and dissemination of *Enterobacteriaceae* isolates producing CTX-M-1–like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. Antimicrob Agents Chemother; 51:796–799.

Nugent, M.E. and Hedges, R.W. (1979). The nature of the genetic determinant for the SHV-1 β-lactamase. Molecular and General Genetics 175: 239–43.

Ogura, T.P. Bouloc, H. Niki, D'Ari, R. Hiraga, S. and Jaffe, A. (1989). Penicillin-binding protein 2 is essential in wild-type *Escherichia coli* but not in *lov* or *cya* mutants. J. Bacteriol. 171:3025–3030.

Oliver, A. Perez-Diaz, J.C. Coque, T.M. Baquero, F. and Canton, R. (2001). Nucleotide sequence and characterization of a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-10) isolated in Spain. Antimicrob. Agents Chemother. 45**:**616–620.

Opazo, A. Sonnevend, A. Lopes, B. Hamouda, A. Ghazawi, A. Pal, T. and Amyes, S.G.B. (2012). Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in Acinetobacter baumannii isolated in the United Arab Emirates. J Antimicrob Chemother; 67: 1619-22.

Oteo, J. Hernández-Almaraz, J.L. Gil-Antón, J. Vindel, A. Fernández, S. Bautista,V. and Campos, J. (2010). Outbreak of VIM- 1-carbapenemase producing

Enterobacter cloacae in paediatric intensive care unit. Pediatr.Infect. Dis.J. 29: 1144–1146.

Otten H. (1986). *Domagk and the development of the sulphonamides.* J Antimicrob Chemother; 17: 689-696.

Queener, S.F. (1986). History and Origins of β-Lactam Antibiotics. In: Queener, SF.; Webber, JA.; Queener, SW., editors. β-Lactam Antibiotics for Clinical Use. New York: Marcel Dekker, Inc.

Quinteros, M. Radice, M. Gardella, N. Rodriguez, M.M. Costa, N. Korbenfeld, D. *et al*. (2003). Extended-spectrum β-lactamases in enterobacteriaceae in Buenos Aires, Argentina, public hospitals. Antimicrob. Agents Chemother**.** 47**:** 2864–2867.

Pagani, L. Migliavacca, R. Pallecchi, L. Matti, C. Giacobone, E. Amicosante, G. Romero, E. Couto, E. Gutkind, G. (2002). Emerging extended-spectrum β-lactamases in *Proteus mirabilis*. J. Clin. Microbiol. 40**:** 1549–1552.

Pagès, J.M. (2004). Role of bacterial porins in antibiotic susceptibility of Gram-negative bacteria in *Bacterial and Eukaryotic Porins* (ed. Benz, R.) 41–59 (Wiley-VCH, Weinheim).

Papp-Wallace, K.M. Bethel, C.R. Distler, A. Kasuboski, C. Taracila, M. and Bonomo, A.A. (2009). Inhibitor resistance in the KPC-2 β-lactamase: a pre-eminent property of this class A β-lactamase. Antimicrob. Agents Chemother. [Epub ahead of print.] doi:10.1128/AAC.00693-09.

Paterson, D.L. (2001). Infection control implications of extended-spectrum β-lactamase (ESBL) production by Klebsiellae and other gram-negative bacteria. Australian Infection Control (AUST INFECT CONTROL); 6: 72-80.

Paterson, D.L. and Bonomo R.A. (2005). Extended-spectrum β-lactamases: a clinical update, Clin. Microbiol. Rev. 18: 657–686.

Paterson, D.L. Hujer, K.M. Hujer, A.M. Yeiser, B. Bonomo, M.D. Rice, L.B. and Bonomo, R.A. (2003). Extended-spectrum β-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type *b*-lactamases. Antimicrob. Agents Chemother. 47**:** 3554–3560.

Paterson, D.L. Ko, W.C. von Gottberg, A. Mohapatra, S. Casellas, M. and Goossens, H. Mulazimoglu, L. Klugman, K.P. Bonomo, R.A.; Rice, L.B. Wagener, M.M. McCormack, J.G. Yu, V.L. and Trenholme, G. (2004. International prospective study of *Klebsiella pneumoniae* bacteriemia: Implications of extended spectrum β- lactamase production in nosocomial infections. Ann. Int. Med. 140: 26-32.

Paulsen, I.T. Brown, M.H. and Skurray, R.A. (1996). Proton-dependent multidrug efflux systems. Microbiol. Rev. 60:575–608.

Payne, D.J. and Amyes, S.G.B. (1991). Transferable resistance to extended-spectrum β-lactams: a major threat or a minor inconvenience? Journal of Antimicrobial Chemotherapy 27, 255–261.

Payne, D.J. Woodford, N. and Amyes, S.G.B. (1992). Characterisation of the plasmid-mediated β-lactamase BIL-1. J.Antimicrob.Chemother. 30:119-127.

Peirano, G. Pitout, J.D. (2010). Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. Int. J. Antimicrob. Agents 35:316–321.

Pérez-Pérez, F.J. and Hanson, N.D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. 40**:**2153-2162.

Perilli, M. Felici, A. Franceschini, N. Santis, A.D. Pagani, L. Luzzaro, F. Oratore, A. Rossolini, G.M. Knox, J.R. and Amicosante, G. (1997). Characterization of a new TEM-derived β-lactamase produced in a *Serratia marcescens* strain. Antimicrob. Agents Chemother. 41**:**2374–2382.

Petroni, A. Corso, A. Melano, R. Cacace, M.L. Bru, A.M. Rossi, A. Galas, M. (2002). Plasmidic extended-spectrum β-lactamases in *Vibrio cholerae* O1 El Tor isolates in Argentina. Antimicrob. Agents Chemother. 46**:** 1462–1468.

Petrosino, J.F. Pendleton, A.R. Weiner, J.H. and Rosenberg, S.M. (2002). Chromosomal system for studying AmpC-mediated β-lactam resistance mutation in *Escherichia coli*. Antimicrob. Agents Chemother. 46**:** 1535–1539.

Philippon A. Arlet G. and Jacoby G.A. (2002). Plasmid-determined AmpC-type *b*-lactamases. Antimicrob. Agents chemother. 46: 1–11.

Picard, B. Sevali Garcia, J. Gouriou S, et al. (1999). The link between phylogeny and virulence in Escherichia coli extraintestinal infection. Infect Immun;67:546-53.

Pilhofer, M. Bauer, A.P. Schrallhammer, M. Richter, L. Ludwig, W. Schleifer, K.H. and Petroni, G. (2007). Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel Two-Step Gene Walking method. Nucl Acids Res, 35:e135.

Pitout, J.D. Campbell, L. Church, D.L. Gregson, D.B. and Laupland, K.B. (2009). Molecular characteristics of travel-related extended-spectrum β-lactamase-producing Escherichia coli isolates from the Calgary Health Region. Antimicrob Agents Chemother. 53: 2539–43.

Pitout, J.D. Church, D.L. Gregson, D.B. Chow, B.L. McCracken, M**. Mulvey, M.R. and Laupland, K.B. (2007).** Molecular epidemiology of CTX-M-producing

Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob Agents Chemother ;51:1281-1286.

Pitout, J.D.D. and Laupland, K.B. (2008). Extended-spectrum β-lactamase producing Enterobacteriaceae; an emerging public health concern. Lancet Infect Dis; 8: 159–66.

Pitout, J.D. Nordmann, P. Laupland, K.B. Poirel, L. (2005). Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. J Antimicrob Chemother; 56:52-59.

Pitton, J.S. (1972). Mechanism of bacterial resistance to antibiotics. Rev. Physiol. 65:15–93.

Poirel, L. Naas, T. and Nordmann, P. (2008). Genetic support of extended-spectrum β-lactamases. Clin. Microbiol.Infect. 14: 75–81.

Poirel, L. Bonnin, R.A. and Nordmann, P. (2011). Analysis of the resistome of a multidrug-resistant NDM-1- producing *Escherichia coli* strain by high-through put genome sequencing. Antimicrob.AgentsChemother. 55: 4224–4229.

Poirel, L. Decousser, J.W. and Nordmann, P. (2003). Insertion sequence ISEcp1B is involved in expression and mobilization of a blaCTX-M b-lactamase gene. Antimicrob Agents Chemother; 47: 2938–45.

Poirel, L. Gniadkowski, M. and Nordmann, P. (2002). Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3. J. Antimicrob. Chemother. 50**:**1031-1034.

Poirel L. and Nordmann P. (2002). Acquired carbapenem-hydrolyzing *b*-lactamases and their genetic support. Curr. Pharm. Biotechnol. 3: 117–127.

Poole, K. (2000). Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria. Antimicrobial Agents and Chemotherapy 44: 2233–2241.

Poole, K. (2004). Review: Resistance to *b*-lactam antibiotics, CMLS, Cell. Mol. Life Sci. 61; 2200–2223.

Potz, N.A.C. Hope, R. Warner, M. Johnson, A.P. and Livermore, D.M. (2006). Prevalence and mechanisms of cephalosporin resistance in Enterobacteriaceae in London and South-East England. Journal of Antimicrobial Chemotherapy, 58: 320-326.

Pournaras, S. Markogiannakis, A. Ikonomidis, A. Kondyli, L. Bethimouti, K. Maniatis, A.N. Legakis, N.J. and Tsakris, A. (2006). Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit. J. Antimicrob. Chemother. 57:557-561.

Pottumarthy, S. Moland, E. Juretschko, S. Sawnzy, S. Thomson, K. and Fritsche, T. (2003). NmcA Carbapenem-hydrolysing Enzyme in *Enterobacter cloacae* in North America. Emerg. Infect Dis. 9: 999-1002.

Prinarakis, E.E. Miriagou, V. Tzelepi, E. Gazouli, M. Tzouvelekis, L.S. (1997). Emergence of an inhibitor-resistant β-lactamase (SHV-10) derived from an SHV-5 variant. Antimicrob. Agents Chemother. 41**:**838–840.

Queenan, A.M. and Bush, K. (2007). Carbapenemases: the versatile β-lactamases. Clin. Microbiol. Rev. 20**:**440-458.

Quinn, J.P. Miyashiro, D. Sahm, D. Flamm, R. and Bush, K. (1989). "Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*," Antimicrobial Agents and Chemotherapy, 33:1451–1456.

Rachakonda, S. and Cartee, L. (2004). Challenges in antimicrobial drug discovery and the potential of nucleoside antibiotics, Curr. Med. Chem. 11: 775-793.

Radice, M. Power, P. Gutkind, G. Fernandez, K. Vay, C. Famiglietti, A. Ricover, N. and Ayala, J. (2004). First class A carbapenemase isolated from *Enterobacteriaceae* in Argentina. Antimicrob. Agents Chemother. 48:1068–1069.

Rasmussen, B.A. Bush, K. Keeney, D. Yang, Y. Hare, R. O'Gara, C. and Medeiros, A.A. (1996). Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob.Agents Chemother. 40**:**2080–2086.

Reinert, R.R. Low, D.E. Rossi, F, *et al*. (2007).Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline. J Antimicrob Chemother;60:1018-29.

Reisbig, M.D. Hossain, A. and Hanson, N.D. (2003). Factors influencing gene expression and resistance for Gram-negative organisms expressing plasmid-encoded *ampC* genes of *Enterobacter* origin. J. Antimicrob. Chemother. 51**:** 1141–1151.

Richards A.N. (1943). Statement released by the Committee on Medical Research. J Am Med Assoc. 122.

Ripoll, A. Baquero, F. Novais, A. Rodríguez-Domínguez, M.J. Turrientes, M.C. Cantón, R. and Galán, J.C. (2011). In vitro selection of variants resistant to β-lactams plus β-lactamase inhibitors in CTX- M β-lactamases: predicting the in vivo scenario? Antimicrob.Agents Chemother. 55: 4530–4536.

Rodriguez-Martinez, J.M. Pascual, A. Garcia, I. and Martinez-Martinez, L. (2003). Detection of the plasmid-mediated quinolone resistance determinant *qnr* among clinical isolates of *Klebsiella pneumoniae* producing AmpC-type β-lactamase.

J. Antimicrob. Chemother. 52**:** 703–706.

Rogers, B.A. Sidjabat, H.E. and Paterson, D.L. (2011). *Escherichia coli* O25b-ST131: apandemic, multiresistant,community-associated strain. J. Antimicrob. Chemother; 66, 1–14.

Rolinson, G. N. (1998). Forty years of β -lactam research. J Antimicrob Chemother 41: 589-603.

Rossolini, G.M. D'Andrea, M.M. and Mugnaioli, C. (2008). Thespread of CTX-M-typeextended-spectrum β-lactamases. Clin. Microbiol.Infect. 14, 33–41.

Rowe-Magnus D.A. and Mazel D. (2002). The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. 292: 115–125.

Rupp, M.E. and Fey, P.D. (2003). Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae: considerations for diagnosis, prevention, and drug treatment. Drugs; 63:353-65.

Ruppé, E. (2010). Epidemiology of expanded-spectrum β-lactamases: the rise of CTX-M. Antibiotiques 12, 3–16.

Russo, T.A. and Johnson, J.R. (2000). Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J Infect Dis;181:1753-1754.

Sabate´, M. Tarrago´, R. Navarro, F. Miró, E, Vergés, C. Barbé, J. and Prats, G. (2000). Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from Escherichia coli in Spain. Antimicrob Agents Chemother; 44: 1970–3.

Saeed, M.Z. Haque, A. Ali, A. Mohsin, M. Bashir, S. Tariq, A. Afzal, A. Iftikhar, T. and Sarwar, Y. (2009). Relationship of drug resistance to phylogenetic groups of *E. coli* isolates from wound infections, J Infect Dev Ctries; 3:667-670.

Saier, M.H. and Paulsen, I.T. (2001). Phylogeny of multidrug transporters. Seminars in Cellular and Developmental Biology 12: 205–13.

Saladin, M. Cao, V.T. Lambert, T. Donay, J.L. Herrmann, J.L. Ould-Hocine, Z. Verdet, C. Delisle, F. Philippon, A. and Arlet, G. (2002). Diversity of CTX-M β-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol. Lett. 209**:**161–168.

Salverda, M.L.M. de Visser, J.A.G.M. Barlow, M. (2010). Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance: review article, FEMS Microbiol Rev 34; 1015–1036.

Schatz, A., E. Bugie, and Waksman S. A., (1944). Streptomycin a substance exhibiting antibiotic activity against gram positive and gram-negative bacteria. Proc. Soc. Exp. Biol. Med. 55**:**66-69.

Schmitz, F.J. Martinez-Freijo P. Theis, S. Fluit, A.C. Verhoef, J. Heinz, H.P. Jones, M.E. (1999). Prevalence of class I integrons and association with decreased antibiotic susceptibility in German gram negative blood culture isolates. Clin. Microbiol. Infect. 5:496–498.

Schwaber, M.J. and Carmeli, Y. (2008). Carbapenem-resistant Enterobacteriaceae: a potential threat. JAMA, 24: 2911-2913.

Schwartz, D.C. Saffran, W. Welsh, J. Haas, R. Goldenberg, M. and Cantor, C.R. (1982). Cold Spring Harbor Symp. Quant. Biol. 47, 198-195.

Segal, H. Nelson, E.C. and Elisha, B.G. (2004). Genetic environment and transcription of *ampC* in an *Acinetobacter baumannii* clinical isolate. Antimicrob. Agents Chemother. 48**:** 612–614.

Shaheen, B.W. Nayak, R. Foley, S.L. Kweon, O. Deck, J. Park, M. Rafii, F. and Boothe, D.M. (2011). Molec- ular characterization of resistance to extended-spectrum cephalosporins in clinical *Escherichia coli* isolates from companion animals in the United States. Antimicrob. Agents Chemother. 55: 5666–5575.

Sherris C.J., Ryan K.J., Ray C.G., Champoux J.J., Neidhardt F.C., Drew W.L., Plorde J.J., Marchalonis J.J., Falkow S., and Robinovitch M.R. (2004). Sherris Medical Microbiology, An Introduction To Infectious Diseases, Gourth Edition, McGRAW-HILL Medical Publishing Division.

Shine, J. and Dalgarno, L. (1974). Identical 3'-terminal octanucleotide sequence in 18S ribosomal ribonucleic acid from different eukaryotes. A proposed role for this sequence in the recognition of terminator codons. Biochem J. 141:609-15.

Silver L.L. (2003). Novel inhibitors of bacterial cell wall synthesis, Curr. Opin. Microbiol.; 6: 431-438.

Sonnevend, A. Al Dhaheri, K. Mag, T. Herpay, M. Kolodziejek, J. Nowotny, N. Usmani, A. Sheikh, F.A. Pál, T. (2006). CTX-M-15-producing multidrug-resistant enteroaggregative Escherichia coli in the United Arab Emirates. Clin Microbiol Infect; 12:582-5.

Sougakoff, W. L'Hermite, G. Pernot, L. Naas, T. Guillet, V. Nordmann, P. Jarlier, V. and Delettre, J. (2002). Structure of the imipenem-hydrolyzing class A β-lactamase SME-1 from *Serratia marcescens*. Acta Crystallogr. D 58**:**267–274.

Spratt, B. G. (1975). Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA 72:2999–3003.

Spratt, B.G. (1977). Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. 72:341–352.

Spratt, B.G. (1980). Deletion of the penicillin-binding protein 5 gene of *Escherichia coli*. J. Bacteriol. 144:1190–1192.

Spratt, B.G. (1994). Resistance to antibiotics mediated by target alterations. Science 264: 388-393.

Spratt, B.G. and A.B. Pardee. (1975). Penicillin-binding proteins and cell shape in *E. coli*. Nature 254:516–517.

Stalder, T. Barraud, O. Casellas, M. Dagot, C. and Ploy, M.C. (2012). Frontiers Research Foundation , 3: 119.

Steward, C.D. Rasheed, J.K. Hubert, S.K. Biddle, J.W. Raney, P.M. Anderson, G.J. Williams, P.P. Brittain, K.L. Oliver, A. McGowan, J.E. and Tenover, F.C. (2001). Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β-lactamase detection methods. J. Clin. Microbiol. 39: 2864–2872.

Stock, I. Sherwood, K.J. and Wiedemann, B. (2003). Natural antibiotic susceptibility of *Ewingella americana* strains. J. Chemother. 15**:** 428–441.

Stock, I. and Wiedemann, B. (2003). Natural antimicrobial susceptibilities and biochemical profiles of *Yersinia enterocolitica*-like strains: *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii* and *Y. rohdei*. FEMS Immunol. Med. Microbiol. 38**:** 139–152.

Sturenburg, E. and Mack, D. (2003). Extended-spectrum β-lactamase: implications for the clinical microbiology laboratory, therapy and infection control. J Infect 2003; 47: 273-95.

Sun, Y. Zeng, Z. Chen, S. Ma, J. He, L. Liu, Y. Deng, Y. Lei, T. Zhao, J. and Liu, J.H. (2010). High prevalence of *bla*(CTX-M) extended-spectrum beta-

lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. Clin Microbiol Infect, 16:1475-81.

Suzuki, H. Nishimura, Y. and Hirota, Y. (1978). On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. Proc. Natl. Acad. Sci. USA 75:664–668.

Swaren, P. Maveyraud, L. Raquet, X. Cabantous, S. Duez, C. Pedelacq, J.D. Mariotte-Boyer, S. Mourey, L. Labia, R. Nicolas-Chanoine, M.H. Nordmann, P. Frere, J.M. and Samama, J.P. (1998). X-ray analysis of the NMC-A β-lactamase at 1.64-Å resolution, a class A carbapenemase with broad substrate specificity. J. Biol. Chem. 273**:**26714–26721.

Sykes, R. (2010). The 2009 Garrod lecture: the evolution of antimicrobial resistance: a Darwinian perspective. J Antimicrob Chemother, 65:1842-1852.

Sykora, P. (1992). Macroevolution of plasmids: a model for plasmid speciation, J. Theor. Biol. 159:53–65.

Tassios, P.T. Gazouli, M. Tzelepi, E. Milch, H. Kozlova, N. Sidorenko, S. Legakis, N.J. and Tzouvelekis, L.S. (1999). Spread of *Salmonella typhimurium* clone resistant to expanded-spectrum cephalosporins in three European countries. J. Clin. Microbiol. 37**:**3774–3777.

Tenover, F.C. Mohammed, J.M. Gorton, T. and Dembek, Z.F. (1999). Detection and reporting of organisms producing extended-spectrum β-lactamases: survey of laboratories in Connecticut. J. Clin. Microbiol. 37:4065–4070.

Tham, J. Odenholt, I. Walder, M. Brolund A, Ahl, J. Melander, E. (2010). Extended-spectrum beta-lactamase-producing Escherichia coli in patients with travellers' diarrhoea. Scand J Infect Dis; 2: 275–80.

Thanassi, D.G. Cheng, L.W. and Nikaido, H. (1997). Active efflux of bile salts by Escherichia coli. J. Bacteriol. 179: 2512–8.

Thomas, C.M. and Nielsen, K.M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3: 711–721.

Tipper, D. J. and Strominger, J. L. (1965). Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci USA 54: 1133-1141.

Tomasz, A. (1980). On the mechanisms of the irreversible antimicrobial effects of β-lactams. Philos. Trans. R. Soc. London Ser. B 289:303–308.

Turner, P.J. Greenhalgh, J.M. Edwards, J.R. and McKellar J. (1999) TheMYSTIC (meropenem yearly susceptibility test information collection) programme. Int. J. Antimicrob. Agents, 13: 117–125.

Tzouvelekis, L. S. and Bonomo, R. A. (1999). SHV-typeβ-lactamases. Curr. Pharm. Des. 5:847–864.

Urban, C. Mariano, N. Bradford, P.A. Tuckman, M. Segal-Maurer, S. Wehbeh,W. Grenner, L. Colon Urban, R. Johnston, B. Johnson, J. R. and Rahal, J.J.(2010). Identification of CTX-M β-lactamases in *Escherichia coli* from hospitalized patients and residents of long term care facilities, Diagn. Microbiol. Infect. Dis. 66: 402–406.

Villegas, M.V. Lolans, K. del Rosario, O.M. Suarez, C.J. Correa, A.Queenan, A.M. and Quinn. J.P. (2006). First detection of metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* isolates from Colombia. Antimicrob. Agents Chemother. 50**:**226–229.

Walther-Rasmussen, J. and Hoiby, N. (2002). Plasmid-borne AmpC β-lactamases. Can. J. Microbiol. 48**:** 479–493.

Webber, M.A. and Piddock, L. J.V. (2003). The importance of efflux pumps in bacterial antibiotic resistance, *Journal of Antimicrobial Chemotherapy;* 51: 9–11.

Wei, Z.Q. Du, X.X. Yu, Y.S. Shen, P. Chen, Y.G. and Li, L.J. (2007). Plasmid-mediated KPC-2 in a *Klebsiella pneumoniae* isolate from China. Antimicrob. Agents Chemother. 51**:**763–765.

Well J.S. Trejo W.H. Bush K. Georpapadakou N.H. Bonner D.P. and Sykes R.B. (1982). EM5400, a family of monobactam antibiotics produced by Agrobacterium radiobacter. I. Taxonomy, fermentation and biological properties. J Antibiotics; 35:295–299.

White D.G. Alekshun M.N. and McDermott P.F. (2005). Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy. ASM Press, Washington,DC.
Wilke, M.S. Lovering, A.L. and Strynadka, N.C. (2005). β-lactam antibiotic resistance: a current structural perspective. Curr Opin Microbiol 8: 525-533.

Winokur, P.L. Canton, R. Casellas, J.M. and Legakis, N. (2001). "Variations in the prevalence of strains expressing an extended-spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region," Clinical Infectious Diseases, 3:294–103.

Woodford, N. (2008). Successful, multiresistant bacterial clones. J. Antimicrob. Chemother. 61:233–234.

Woodford, N. Carattoli, A. Karisik, E. Underwood, A. Ellington, M.J. and Livermore, D.M. (2009). Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-Menzymes in three major *Escherichia coli*

lineages from the United Kingdom, all belonging to the international O25:H4- ST131 clone. Antimicrob. Agents Chemother. 53, 4472–4482.

Woodford, N., Fagan, E. J. and Ellington, M. J. (2006). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum (β)-lactamases. J Antimicrob Chemother, 57(1):154-5.

Woodford, N. Turton, J.F. and Livermore, D.M. (2011). Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol.Rev. 35, 736–755.

Woodford, N. Payne D. J. Johnson, A.P. Weinbren, M.J. Perinpanayagam, R. M. George, R.C. Cookson, B.D and Amyes, S.G.B. (1990). Transferable cephalosporin resistance not inhibited by clavulanate in *Escherichia coli*. Lancet 336:253.

Woodford, N. Ward, M. E. Kaufmann, M. E. Turton, J. Fagan, E. J. James, D. Johnson, A. P. Pike, R. Warner, M. Cheasty, T. Pearson, A. Harry, S. Leach, J. B. Loughrey, A. Lowes, J. A. Warren, R. E. and Livermore, D. M. (2004). Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. J. Antimicrob. Chemother. 54**:**735-743.

Wright G.D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification, Adv. Drug Deliv. Rev., 57: 1451-1470.

Yang, Y. Wu, P. and Livermore, D. M. (1990). Biochemical characterization of a β-lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. Antimicrob. Agents Chemother. 34**:**755–758.

Yigit, H. Queenan, A.M. Anderson, G.J. Domenech-Sanchez, A. Biddle, J.W. C. D. Steward, C.D. Alberti, S Bush, K. and Tenover, F.C. (2001). Novel

carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 45**:**1151–1161.

Yoneyama H. and Katsumata R. (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development, Biosci. Biotechnol. Biochem., 70:1060-1075.

Yong, D. Shin J.H. Kim S. Lim Y. Yum J.H. Lee K. Chong, Y.S. Bauernfeind, A. (2003). High prevalence of PER-1 extended-spectrum *b*-lactamase-producing *Acinetobacter* spp. in Korea. Antimicrob. Agents Chemother. 47**:** 1749–1751.

Yousif, S.Y. Broome-Smith, J.K. and Spratt, B.G. (1985). Lysis of *Escherichia coli* by b-lactam antibiotics: deletion analysis of the role of penicillin binding proteins 1A and 1B. J. Gen. Microbiol. 131:2839–2845.

Yu, F.Y. Chen, Q.A. Yu, X.J. Li, Q.Q. Ding, B.X. Yang, L.H. Chen, C. Qin, Z.Q. Parsons, C. Zhang, X.Q. Huang, J.W. Luo, Y. Wang, L.X. Pan, J.Y. (2011). High Prevalence of Extended-Spectrum Beta Lactamases among Salmonella enterica Typhimurium Isolates from Pediatric Patients with Diarrhea in China. PLOS ONE**:** 6: 3.

Yu, Y. JI, S. Chen, Y. Zhou, W. Wei, Z. Li, L. and Ma, Y. (2007). Resistance of strains producing extended-spectrum beta-lactamases and genotype distribution in China. J Infect, 54:53-7.

ZEBA, B. (2005). Review: Overview of b-lactamase incidence on bacterial drug Resistance, African J. Biotech 4: 1559-1562.

Zhao, S. Qaiyumi, S. Friedman, S. Singh, R. Foley, S.L. White, D.G. McDermott, P.F. Donkar, T. Bolin, C. Munro, S. Baron, E.J. and Walker, R.D. (2003). Characterization of *Salmonella enterica* serotype new port isolated from humans and food animals. J. Clin. Microbiol. 41**:** 5366–5371.

Zheng, H. Zeng, Z., Chen, S. Liu, Y. Yao, Q. Deng, Y. Chen, X., Lv, L. Zhuo, C. Chen, Z. and Liu, J.H. (2012). Prevalence and characterisation of CTX-M beta-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. International journal of antimicrobial agents, 39:305-10.

Zong, Z. Partridge, S.R. and Iredell, J.R. (2010). ISEcp1-Mediated Transposition and Homologous Recombination Can Explain the Context of $bla_{CTX\text{-}M\text{-}62}$ Linked to qnrB2. American Society of Microbiology; 54:3039-3042.

8. Appendix 1

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
1	*E. coli*	0.06	0.03	0.12	0.5	0.03	0.03	1	0.5
2	*E. coli*	0.06	0.03	0.12	0.5	0.03	0.06	1	0.5
3	*E. coli*	0.25	0.06	0.03	0.25	0.008	0.06	32	16
4	*E. coli*	0.06	0.06	0.03	0.5	0.008	0.12	0.5	8
5	*E. coli*	0.06	0.06	0.12	0.06	0.015	0.12	8	0.5
6	*E. coli*	0.06	0.12	0.25	0.06	0.015	0.008	8	1
7	*E. coli*	0.06	0.12	0.25	0.25	0.015	0.008	1	1
8	*E. coli*	0.06	0.06	0.25	0.5	0.008	0.008	0.5	1
9	*E. coli*	64	32	8	0.5	0.015	0.015	8	8
10	*E. coli*	32	32	8	0.5	0.12	0.015	8	8
11	*E. coli*	0.06	0.03	0.06	0.5	0.25	0.015	64	8
12	*E. coli*	64	128	16	0.5	0.008	0.06	8	16
13	*E. coli*	0.06	0.12	0.12	0.03	0.008	0.06	32	32

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
14	*E. coli*	0.06	0.25	0.12	0.03	0.015	0.03	0.25	32
15	*E. coli*	0.12	0.06	0.12	0.06	0.12	0.06	0.5	16
16	*E. coli*	0.06	0.06	0.12	0.12	0.12	0.008	1	1
17	*E. coli*	0.06	0.06	0.25	0.12	0.12	0.12	1	0.5
18	*E. coli*	0.06	0.25	0.25	0.06	0.12	0.015	0.5	1
19	*E. coli*	0.06	0.06	0.25	0.5	0.008	0.015	0.25	16
20	*E. coli*	0.06	0.06	0.5	0.5	0.008	0.12	8	0.5
21	*E. coli*	0.06	0.03	0.12	0.12	0.015	0.25	8	1
22	*E. coli*	0.06	0.25	0.12	0.12	0.03	0.5	16	8
23	*E. coli*	64	128	8	0.03	0.12	0.008	16	16
24	*E. coli*	0.12	0.06	0.06	0.03	0.06	0.008	1	1
25	*E. coli*	128	128	64	0.03	0.06	0.008	8	16
26	*E. coli*	0.12	0.12	0.06	0.03	0.06	0.06	8	0.5
27	*E. coli*	0.25	0.015	0.12	0.06	0.12	0.06	16	16
28	*E. coli*	0.06	0.015	0.12	0.06	0.12	0.06	128	1

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
29	*E. coli*	0.06	0.06	0.12	0.03	0.008	0.03	8	8
30	*E. coli*	0.5	0.06	0.06	0.5	0.12	0.015	1	8
31	*E. coli*	0.06	0.25	0.5	0.5	0.25	0.015	1	32
32	*E. coli*	0.06	0.03	0.25	0.5	0.008	0.008	1	16
33	*E. coli*	0.06	0.03	0.25	0.5	0.015	0.06	0.5	16
34	*E. coli*	0.06	0.06	0.12	0.06	0.015	0.015	0.25	16
35	*E. coli*	0.06	0.12	0.12	0.5	0.12	0.5	1	1
36	*E. coli*	0.06	0.25	0.12	0.25	0.25	0.008	0.25	0.5
37	*E. coli*	0.06	0.12	0.12	0.12	0.008	0.06	0.5	0.5
38	*E. coli*	0.12	0.06	0.12	0.06	0.015	0.03	0.5	64
39	*E. coli*	0.25	0.06	0.12	0.03	0.015	0.03	0.5	32
40	*E. coli*	0.06	0.03	0.06	0.03	0.03	0.008	1	8
41	*E. coli*	0.06	0.03	0.06	0.06	0.12	0.015	32	8
42	*E. coli*	0.06	0.06	0.12	0.06	0.008	0.015	16	8
43	*E. coli*	0.12	0.06	0.12	0.06	0.008	0.12	8	1

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
44	*E. coli*	0.03	0.06	0.12	0.03	0.06	0.5	16	1
45	*E. coli*	0.12	0.12	0.12	0.5	0.12	0.12	32	1
46	*E. coli*	0.06	0.03	0.25	0.5	0.5	0.008	1	0.5
47	*E. coli*	0.06	0.03	0.25	0.25	0.03	0.015	1	32
48	*E. coli*	0.06	0.03	0.25	0.03	0.06	0.015	1	32
49	*E. coli*	0.06	0.06	0.25	0.06	0.008	0.06	1	16
50	*E. coli*	0.06	0.25	0.06	0.5	0.12	0.06	0.5	8
51	*K. pneumoniae*	0.03	0.06	0.12	0.5	0.25	0.03	0.5	8
52	*K. pneumoniae*	0.03	0.03	0.12	0.03	0.03	0.03	0.25	8
53	*K. pneumoniae*	0.06	0.12	0.12	0.12	0.06	0.008	1	1
54	*K. pneumoniae*	0.06	0.25	0.12	0.12	0.12	0.12	1	1
55	*K. pneumoniae*	0.03	0.03	0.12	0.03	0.12	0.008	1	1
56	*K. pneumoniae*	0.03	0.015	0.25	0.06	0.25	0.015	32	0.5
57	*K. pneumoniae*	0.03	0.015	0.06	0.5	0.008	0.06	8	0.5
58	*K. pneumoniae*	0.06	0.015	0.25	0.5	0.015	0.06	1	0.5

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
59	*K. pneumoniae*	0.06	0.06	0.5	0.5	0.03	0.008	0.5	8
60	*K. pneumoniae*	128	128	128	0.06	0.06	0.03	8	16
61	*K. pneumoniae*	0.06	0.06	0.12	0.06	0.12	0.03	1	8
62	*K. pneumoniae*	0.03	0.06	0.12	0.03	0.008	0.06	0.5	8
63	*K. oxytoca*	0.03	0.06	0.12	0.12	0.008	0.06	0.5	1
64	*K. oxytoca*	0.03	0.06	0.12	0.03	0.06	0.008	0.25	1
65	*K. oxytoca*	0.06	0.25	0.25	0.5	0.5	0.008	1	1
66	*K. oxytoca*	0.06	0.06	0.25	0.06	0.008	0.12	1	0.5
67	*K. oxytoca*	0.12	0.06	0.12	0.5	0.015	0.015	1	0.5
68	*E. cloacae*	0.03	0.12	0.12	0.5	0.015	0.015	8	64
69	*E. cloacae*	0.03	0.25	0.12	0.5	0.12	0.015	8	64
70	*E. cloacae*	0.03	0.015	0.12	0.12	0.008	0.015	32	32
71	*E. cloacae*	0.03	0.03	0.25	0.25	0.06	0.008	1	16
72	*E. cloacae*	0.25	0.015	0.5	0.25	0.03	0.12	8	16
73	*E. cloacae*	0.25	0.015	0.5	0.25	0.015	0.03	8	16

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
74	*E. cloacae*	0.25	0.06	0.5	0.25	0.06	0.06	16	32
75	*E. cloacae*	0.06	0.03	0.12	0.25	0.06	0.008	16	32
76	*E.*spp	0.25	0.03	0.12	0.5	0.06	0.008	16	1
77	*P. mirabilis*	0.12	0.06	0.12	0.5	0.03	0.008	1	1
78	*P. mirabilis*	0.03	0.06	0.12	0.06	0.008	0.06	1	0.5
79	*P. mirabilis*	0.03	0.06	0.12	0.06	0.12	0.008	0.5	0.5
80	*P. mirabilis*	0.03	0.03	0.25	0.06	0.015	0.008	0.5	0.5
81	*P. mirabilis*	0.03	0.015	0.25	0.03	0.008	0.008	0.5	1
82	*P. mirabilis*	0.03	0.12	0.25	0.03	0.06	0.06	1	1
83	*P. mirabilis*	0.03	0.12	0.25	0.06	0.03	0.015	1	8
84	*P. mirabilis*	0.03	0.12	0.12	0.5	0.03	0.015	1	8
85	*P. mirabilis*	0.06	0.06	0.12	0.12	0.06	0.008	0.5	16
86	*P. mirabilis*	0.06	0.06	0.5	0.12	0.008	0.03	0.25	32
87	*P. mirabilis*	0.12	0.06	0.5	0.12	0.008	0.06	0.25	32
88	*P. mirabilis*	0.12	0.015	0.12	0.03	0.008	0.06	1	32

Isolate No.	Strain	MIC(mg/L)							
		Cefotaxime	Ceftriaxone	Ceftazidime	Ertapenem	Imipenem	Meropenem	Gentamicin	Ciprofloxacin
89	*P. vulgaris*	0.06	0.03	0.12	0.5	0.015	0.06	1	1
90	LFC	0.06	0.03	0.12	0.5	0.12	0.03	0.5	1
91	LFC	0.03	0.03	0.12	0.5	0.5	0.008	8	1
92	LFC	0.06	0.12	0.06	0.5	0.03	0.015	8	0.5
93	LFC	0.06	0.12	0.06	0.06	0.06	0.12	16	0.5
94	LFC	0.06	0.12	0.06	0.03	0.015	0.5	8	0.5
95	LFC	0.06	0.03	0.25	0.06	0.015	0.015	4	8
96	*Pantoea* spp	0.06	0.06	0.12	0.12	0.008	0.06	1	8
97	*Pantoea* spp	0.03	0.06	0.12	0.5	0.06	0.06	1	16
98	*Morganella morganii*	0.03	0.06	0.12	0.5	0.015	0.06	1	8
99	*Hafnia alvei*	0.06	0.06	0.12	0.06	0.03	0.06	1	1
100	*Serratia marcescens*	0.03	0.06	0.12	0.5	0.03	0.03	1	0.5